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The development of inhibition in the neonatal dorsal horn

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Declaration

The work presented in this thesis was conducted in the Department of Anatomy and Developmental Biology at University College London. I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Spinal nociceptive processing undergoes extensive maturation in the postnatal period. Dorsal horn neurons are more easily excited by peripheral stimuli in young animals and nociceptive reflexes are less well directed. Since circuits in the central nervous system require a balance of excitation and inhibition, I hypothesised that the maturation of inhibitory circuitry is responsible for changing nociceptive processing in postnatal animals. To test this I have investigated the maturation of segmental and descending inhibition in the anaesthetised and decerebrate rat dorsal horn using *in vivo* electrophysiological methods.

First, I examined the developmental regulation of inhibitory signalling in spinal nociceptive circuits. Single cells in the lumbar dorsal horn, characterised by their responses to hindpaw cutaneous stimulation, were recorded at two postnatal ages over a prolonged period in the presence and absence of a $GABA_AR$ antagonist. The results show that at the circuit level, GABAergic signalling does not change between postnatal days 3 and 21 and that blocking GABA activity is equally excitatory at both ages.

Next, I examined the role of descending activity from supraspinal centres upon single dorsal horn cell activity at different ages. The results show that while descending inhibition of spinal nociceptive activity is present in young animals, there is also an excitatory descending influence upon young dorsal horn cells that is not observed in older animals.

Finally, I mapped the spatial pattern of inhibitory receptive fields at different ages. The results show that contralateral inhibitory fields are less spatially restricted in young animals and are activated by both low and high intensity stimulation. A model is proposed to show how inhibitory receptive field organisation may determine higher-level sensory processing.

In conclusion, the postnatal maturation of spinal nociceptive processing is likely due to maturation of local and descending inhibitory activity within spinal circuits.

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Abbreviations

AP	action potential
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate
ANOVA	analysis of variance
AUC	area under the curve
CGRP	calcitonin gene-related peptide
CNS	central nervous system
Ca^{2+}	calcium ion
Cl^-	chloride ion
$[Cl^-]$	concentration of chloride ions
DNIC	diffuse noxious inhibitory control
DLF	dorsolateral funiculus
E_{Cl}	chloride reversal potential
E_{GABA}	GABA reversal potential
ECG	electrocardiogram
EMG	electromyograph
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_ARs$	γ-aminobutyric acid receptors with channel subunits in the A group
$GABA_BRs$	γ-aminobutyric acid receptors with channel subunits in the B group
GAD	glutamic acid decarboxylase
GAD65	smaller isoform of glutamic acid decarboxylase
GlyRs	glycine receptors
GPCRs	G protein-coupled receptors
5-HT	5-hydroxytryptamine
IPSC	inhibitory postsynaptic current

IPSP	inhibitory postsynaptic potential
K^+	potassium ion
KCC2	potassium chloride co transporter
kDa	kiloDalton
L4	fourth lumbar segment of the spinal cord
LSO	lateral superior olive
LT	low threshold
LTP	long term potentiation
mEPSCs	mini excitatory postsynaptic currents
mRNA	messenger ribonucleic acid
MK-801	a noncompetitive blocker of glutamate N-methyl-D-aspartate receptors
MNTB	medial nucleus of the trapezoid body
NA	noradrenaline
NKCC1	sodium potassium chloride co transporter
NRM	nucleus raphe magnus
NS	noxious specific
P	postnatal day
PAD	primary afferent depolarization
PCA	postconceptional age
RF	receptive field
RVM	rostral ventromedial medulla
SEM	standard error of the mean
STDP	spike time dependent plasticity
vFh	von Frey hair
VLF	ventrolateral funiculus
WDR	wide dynamic range

Chapter 1

Introduction

1.1 Introduction

Primary afferents carrying tactile and nociceptive information from the peripheral tissues in the body synapse first in the dorsal horn of the spinal cord. Here, the signal is transformed by networks of interneurons before being projected to higher centres in the central nervous system (CNS). The dorsal horn also receives many descending projections from the brainstem which contribute to the complexity of processing in this region.

Inhibition is a vital component of the circuitry in the mature dorsal horn, acting to control the overall "gain" of the signal as well as to modulate it in more subtle ways. In the adult, experimental loss of inhibition is characterised by a reduction in mechanical thresholds (Sivilotti and Woolf 1994; Ishikawa et al. 2000), and there is evidence for disinhibition as a contributing factor in neuropathic and inflammatory pain states (Moore et al. 2002; Coull et al. 2003; Harvey et al. 2004; Zeilhofer 2005). An enhancement of elements of intrinsic inhibitory processing throughout the CNS is also a key aspect of the mechanism of action of some anaesthetic agents, such as etomidate and propofol (Franks 2006), and of the commonly used sedative benzodiazepine (Haefely 1988).

In neonates, however, the circuitry in the dorsal horn is still maturing and, together with other developmental differences, there are indications of a deficit in inhibition. This could potentially have repercussions for the treatment of infants and children undergoing painful procedures: for example, analgesics which work effectively in adults may not necessarily do so in these patient groups. This could be particularly important for premature infants in intensive care, who receive many painful procedures and who cannot verbalise their pain. In addition, traumatic wounds or surgical interventions at an early age may interfere with the ongoing development of the circuitry, and lead to abnormal sensory processing in adulthood (Anand 2000; Peters et al. 2005). In order to address such issues fully, it is necessary to understand how the underlying inhibitory circuitry alters during postnatal development, and the experiments in this thesis were designed with this aim in mind.

The following three sections of this chapter provide an overview of the circuitry and inhibitory mechanisms in the adult dorsal horn. Section 1.5 then details the evidence for a deficit in inhibition in the neonate, and section 1.6 examines the developmental neuroanatomy of the dorsal horn, and the changes which are already known to take place in inhibitory processing during the postnatal period. The final section of this chapter details the aims of the thesis.

1.2 Anatomy and circuitry of the adult dorsal horn

1.2.1 Lamina structure

The dorsal horn of the spinal cord has a distinctive structure composed of six approximately parallel laminae, first identified by Rexed (1952; see figure 1.1). Each lamina has a particular pattern of primary afferent innervation (see table 1.1) and descending input which influences the response properties of cells within that lamina. Moreover, the output from the dorsal horn to the brain is also highly organised according to the laminar location of the projection cells.

Lamina I, also known as the marginal layer, and lamina II, also known as the substantia gelatinosa (SG), are thought to be of particular importance in the processing of noxious information as they are the termination zones for peptidergic and non-peptidergic C fibre nociceptors and Aδ small myelinated nociceptors. Together, laminae I and II are known as the superficial dorsal horn. Lamina I is composed of a minority of large cells with wide-ranging horizontal dendrites (Waldeyer cells) and many smaller neurons, which also tend to arborise in a horizontal fashion within the lamina. Some neurons in lamina I express the neurokinin 1 (NK1) receptor, which is activated by substance P released from the peptidergic C fibre afferents that terminate in this lamina. Around 10% of lamina I neurons have an ascending projection to the brain, of which ~80% are NK1-positive (Todd et al. 2000). The lamina I ascending pathway projects to a number of supraspinal centres, notably the brainstem

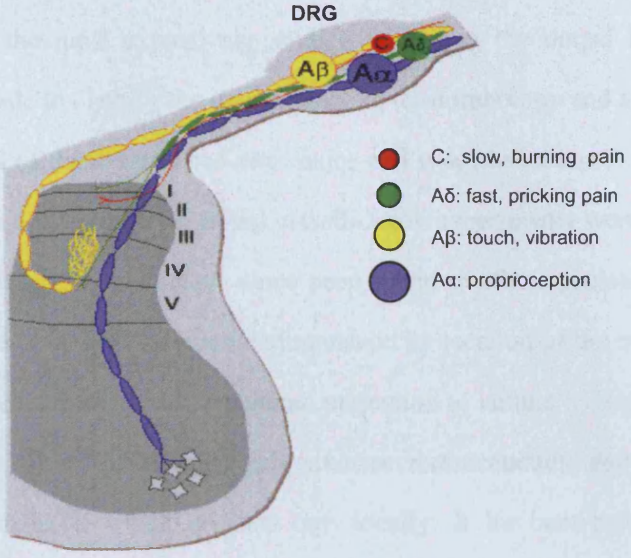


Figure 1.1 Diagram of the adult spinal cord illustrating the lamina structure of the dorsal horn and the termination patterns of the different primary afferent fibre types.

parabrachial area and the periaqueductal grey (PAG), which are linked to limbic and autonomic centres such as the amygdala and the hypothalamus (Hunt and Mantyh, 2001).

These circuits suggest that the lamina I ascending projection may be involved with homeostatic regulation, the affective components of pain, and the production of appropriate pain behaviours, such as the classic "fight or flight" response (Craig 1996, 2002; Hunt and Mantyh 2001). Recent studies also suggest that the lamina I projection forms the ascending branch of a dorsal horn – supraspinal – dorsal horn loop which can set the excitability level of neurons in deeper laminae (Suzuki et al. 2002).

Lamina II, by contrast, contains few if any projection neurons and is composed of interneurons with small cell bodies and the terminals of peptidergic (lamina II_{outer}) and non-peptidergic (IB4+; lamina II_{inner}) C fibre afferents. Some primary afferent terminals in lamina

II form distinctive glomeruli: terminals surrounded by a complex of dendrites, with the whole formation often partially enclosed by glia. The excitatory and inhibitory interneurons of lamina II have been the most extensively studied of any in the dorsal horn, and several attempts have been made to classify the cells according to morphology and axonal projections. The most widely used of these identified two major cell types in lamina II: stalked cells and islet cells (Gobel 1978). Although the initial classification experiments were conducted in the cat, stalked and islet lamina II cells have since been found in other species, including the rat (Todd and Lewis 1986). Stalked cells are distinguished by location of the cell body in lamina II_{outer}, ventrally directed dendrites, and an axonal projection to lamina I. Islet cells are located throughout lamina II, have dendrites which arborise rostrocaudally and which can form synapses, and have an axon which projects only locally. It has been proposed that stalked

Fibre type	Myelin--ated?	Size	Type	Lamina termination	Releases	Expresses
Aβ	Y	large	low-threshold mechanoreceptor, some nociceptors	III - VI	glutamate	Trk C p75
Aδ	Y	small	nociceptor	I, II, V	glutamate	Trk C p75
C peptidergic	N	small	nociceptor	I, II_{outer}	glutamate CGRP substance P	Trk A p75
C non-peptidergic	N	small	nociceptor	II_{inner}	glutamate	Ret GFRα $P2X_3$ *(marked by IB4)*

Table 1.1 Properties of the primary afferent fibres which terminate in the dorsal horn.

cells correspond to excitatory interneurons and islet cells to inhibitory interneurons, a position supported by the finding that islet cells contain γ-aminobutyric acid (GABA), one of the major inhibitory neurotransmitters in the spinal cord (Spike and Todd 1992). However, many cells do not correspond to either stalked or islet cells (Todd and Lewis 1986) and, moreover, attempts to correlate morphology with cell function have often failed to find a clear link between the two (Light et al 1979; Woolf and Fitzgerald 1983; Light and Kavookjian 1988; Rethelyi et al. 1989).

Laminae III - VI together form the deep dorsal horn and are the termination zone for large myelinated Aβ primary afferents, which are primarily activated by low-threshold cutaneous stimulation. A subset of Aβ afferents which carry nociceptive information has recently been identified (see Lawson 2002). Less is known about the interneurons in these laminae, but many have large dendritic arborisations projecting dorsally, which could enable monosynaptic contacts with C fibre terminals in the superficial dorsal horn, as well as with lamina II interneurons (see Willis and Coggeshall 1991). The deep dorsal horn, particularly lamina V, is the source of a second major ascending projection; however, these projection neurons cannot as yet be distinguished from interneurons by immunocytochemistry. The projection cells of the deep dorsal horn send a dense projection to reticular nuclei, including the subnucleus reticularis dorsalis (SRD) and the gigantocellular nuclei (NGc), which in turn project to the thalamus (Gauriau and Bernard 2002). It has long been proposed that the lamina V projection system subserves the discriminatory aspects of pain perception, a hypothesis supported by the finding that selective destruction of the lamina I projection system leaves detection of acute noxious stimuli unimpaired (Mantyh et al. 1997). This neat division of function between superficial and deep ascending pathways is of course an oversimplification as there is a large degree of overlap in projection targets, as well as many polysynaptic connections between the different supraspinal regions known to be associated with one or other projection.

1.2.2 Functional classification of dorsal horn neurons

1.2.2.1 Excitatory receptive fields

Neurons within the dorsal horn can be assessed according to their cutaneous receptive field (RF). A cutaneous RF is the spatial region on the skin surface which, when stimulated, alters the firing of the neuron; figure 1.2 shows a diagrammatic representation of this. The centre of the RF is usually the most sensitive and the maximum possible limits of the excitatory RF are delineated by the connections of the recorded cell with primary afferent fibres. These could be direct and monosynaptic or polysynaptic via excitatory interneurons. Similarly, the set of stimulus modalities to which a cell can respond is fundamentally determined by the primary afferent monosynaptic or polysynaptic input to that cell. For example, if a cell only receives Aβ fibre input, then it will respond only to low-threshold, innocuous stimulation, such as brush or touch.

Dorsal horn cells have for some years been divided into three main categories based upon the responses to mechanical stimuli within the RF: (i) low-threshold mechanoreceptors (LT; class 1) which respond only to brush or innocuous touch, or both; (ii) wide dynamic range cells (WDR; class 2) which respond in a graded fashion to both innocuous (brush, touch) and noxious stimuli; and (iii) nociceptive specific cells (NS; class 3) which are driven only by noxious stimuli, such as pinch or pin-prick. Various cell classifications (including the WDR category) had previously been proposed from studies undertaken in the cat (Mendell 1966; Price and Browe, 1973) and monkey (Price and Mayer 1974), but these three defining classes were first described in the rat by Menetrey et al. (1977), who also subdivided them and listed a fourth class of cells which responded to joint movement or deep pressure.

In the rat dorsal horn the majority of cells recorded both intracellularly and extracellularly are WDR, with fewer LT and NS cells (Menetrey et al. 1977; Woolf and Fitzgerald 1983, 1986). The proportion of NS cells is greatest in the superficial laminae, especially lamina I, whereas WDR cells are most commonly found in deeper laminae, especially lamina V (Menetrey et al.

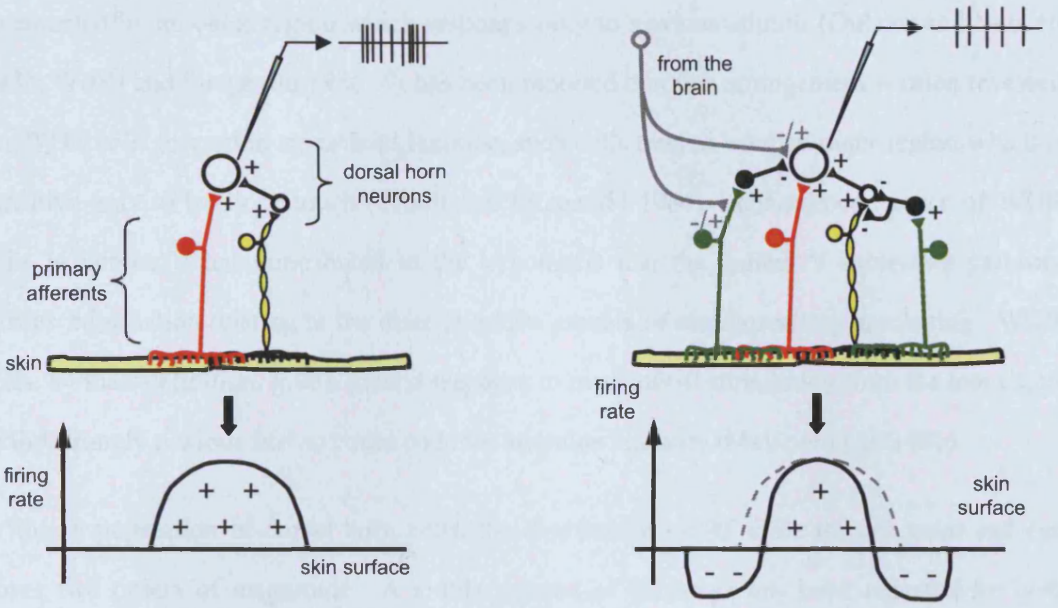


Figure 1.2 Diagrammatic representation of cutaneous receptive fields (RF)
The excitatory RF of a second-order dorsal horn neuron depends critically on the monosynaptic and polysynaptic connections with primary afferent fibres (left). RFs may also have an inhibitory component, which can be mediated by inhibitory interneurons (black) or descending fibres (grey) (right; see text for more details). In some cells, the inhibition may form the classic "Mexican hat" spatial surround illustrated here (lower right).

1977; Woolf and Fitzgerald 1986). This correlates well with the known pattern of primary afferent termination described above; however, all three classes of cell are found with reasonable frequency in all laminae, so the modality responsiveness of any one neuron cannot be predicted from its laminar location (Woolf and Fitzgerald 1983, 1986). Transcutaneous electrical stimulation allows identification of the afferent input to a cell: input from myelinated A fibres can be seen ~ 10 - 50 ms after the stimulation, whereas the input from small unmyelinated C fibres is seen later, at ~150 - 300 ms. This method has been used to show that a large proportion of dorsal horn cells receive both A and C fibre input (Menetrey et al. 1977), which is consistent with the high incidence of WDR cells. WDR cells tend to have large RFs compared with LT and NS cells and commonly have a composite structure

comprising a central region which is sensitive to both noxious and innocuous stimuli, surrounded by an outer region which responds only to noxious stimuli (Dubner and Bennett 1983; Woolf and Fitzgerald 1986). It has been reported that this arrangement is often reversed for WDR cells located in superficial laminae; such cells tend to have an outer region which is sensitive only to brush or touch (Woolf and Fitzgerald 1986). The preponderence of WDR cells in lamina V has contributed to the hypothesis that the lamina V ascending pathway carries information relating to the discriminative aspects of somatosensory processing. WDR cells, by their definition, give a graded response to mechanical stimulation from the innocuous to the strongly noxious and so could code for stimulus intensity (Maixner et al. 1986).

Within a population of dorsal horn cells, the distribution of RF sizes is very great and can cover two orders of magnitude. A similar spread of RF areas has been reported for both superficial and deep cells, with the exception of a subpopulation of superficial cells with unusually large RFs (Woolf and Fitzgerald 1986). The RFs of superficial dorsal horn cells have also been demonstrated to be dynamic, with a proportion (~10%) in both the rat and the cat found to vary in size or modality of effective stimulation, or both, during the course of the recording (Dubuisson et al. 1979; Woolf and Fitzgerald 1983).

Dorsal horn cell responses in all species tested also vary along dimensions other than those outlined above. For example, some cells, particularly in superficial laminae, habituate to repeated stimuli whereas others show a prolonged afterdischarge in response to a single, usually noxious, stimulus (Hentall 1977; Light et al. 1979; Woolf and Fitzgerald 1983). These different properties, together with the diversity in RF size and cell classification, illustrate the heterogenous nature of neurons throughout the dorsal horn.

1.2.2.2 The role of inhibition in shaping receptive fields

Networks of inhibition in the dorsal horn and from supraspinal centres play a key role in shaping the RF size and responsiveness to stimuli, within the fundamental limits determined by the excitatory input from primary afferents. As illustrated in figure 1.2, neighbouring

primary afferents may act via inhibitory interneurons to sharpen the excitatory RF for a particular cell or to inhibit input from certain modalities. In some cells, an inhibitory RF may surround the excitatory RF, to produce classic centre-surround "Mexican hat" RFs (Hillman and Wall 1969; Woolf and Fitzgerald 1983; see figure 1.2b). The interneurons could directly inhibit the postsynaptic recorded cell, or could act indirectly on intermediary interneurons or on primary afferent terminals (see section 1.3.1.1 below). The latter inhibitory action, known as primary afferent depolaristion (PAD), provides a parsimonious mechanism for selective inhibition of a specific modality: one can envisage a simple circuit whereby one set of primary afferent terminals only is subject to this presynaptic inhibition (Cervero and Laird 1996). In addition to the inhibitory action of local interneurons, the dorsal horn is also influenced by descending inhibition and facilitation, which can serve to modulate the size, specificity and sensitivity of RFs (see section 1.4 below).

What is the purpose of this inhibitory control? If smaller excitatory receptive fields are beneficial, it would be simpler to wire them that way initially instead of tuning them with inhibition. However, this would produce a rather rigid system, without the following benefits provided by inhibitory control: first, inhibition is necessary to balance the excitatory input and act as a gain control mechanism for the dorsal horn as a whole, otherwise useful information would be lost during periods of intense activity. Second, the sharpening of excitatory RFs by inhibitory mechanisms produces a flexible system in which the size, sensitivity and modality specificity of individual excitatory RFs are not fixed but can be modified over time and in response to the needs of the system. RFs can also be changed by altering the excitatory inputs, as is known to occur in central sensitisation (Woolf and Salter 2000) but inhibition provides an elegant and rapid means of control. Third, a surround inhibitory RF conceptually enables a cell to encode more information: in theory, a cell with a centre-surround RF organisation could filter out stimuli with a broad spatial profile and increase the contrast in firing produced by stimuli located inside and outside the excitatory RF (see figure 1.3). This "feature-extraction" function of centre-surround RFs is well documented in the visual and auditory systems (Allman et al. 1985; Suga 1995), but it is less

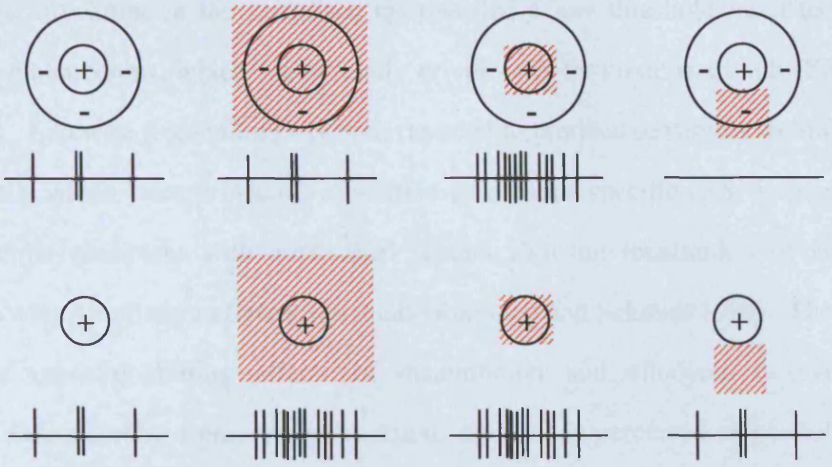


Figure 1.3 Idealised receptive fields (RFs) and firing patterns for a cell with an inhibitory surround RF (top) and a cell with a small excitatory RF only (bottom). Red hatched areas indicate stimulation applied to skin surface. The centre-surround organisation allows greater distinction between the different types of stimulus shown.

certain whether this it is an essential function of the inhibitory networks in the dorsal horn. Fourth, descending inhibition (and facilitation) of the dorsal horn from the brain is an important adaptive mechanism enabling environmental context to influence the initial processing of somatosensory stimuli and aid in the appropriate behavioural response.

There is strong experimental evidence to support the role of inhibition in shaping both the spatial input and modality selectivity of dorsal horn RFs. The application of bicuculline (an antagonist at the $GABA_A$ receptor) to the spinal cord during *in vivo* extracellular recordings caused an increase in RF area in rats (Drew et al. 2004; Kawamata et al. 2005), suggesting a tonic control of RF size by GABA. Application of strychnine (a glycine receptor antagonist) in anaesthetised cats caused an increase in the receptive fields of LT cells only (Sorkin and Puig 1996). In the rat, strychnine unmasked input from the saphenous nerve to cells which previously were responsive only to sciatic stimulation (Biella and Sotgiu 1995), signifying

that glycine can also mediate spatial control of excitatory RFs. Spinal application of either bicuculline or strychnine in the spinalised rat revealed a low threshold input to hamstring flexor alpha motoneurons, which are normally driven only by noxious stimuli (Sivilotti and Woolf 1994). Likewise, bicuculline has been reported to produce sensitivity to low threshold stimuli in cells which were previously classified as noxious specific (NS; Kawamata et al. 2005), which is consistent with anatomical reports showing localisation of GABAergic interneurons with Aβ primary afferent terminals (Rudomin and Schmidt 1999). The similarity between the modality-shifting effects of disinhibition and allodynia (a symptom of neuropathic pain whereby a previously innocuous stimulus is perceived as painful) have led some to propose disinhibition as an underlying factor in neuropathic pain (Sivilotti and Woolf 1994).

1.3 Intrinsic inhibitory neurotransmitters in the dorsal horn

As shown in figure 1.2, inhibition can be mediated in a number of distinct ways, including: the classical postsynaptic opening of ligand-gated anion channels; the activation of metabotropic G-protein coupled receptors (GPCRs); presynaptic inhibition of primary afferent terminals; and descending inhibition via serotonergic and noradrenergic fibres. Within the spinal cord, the major inhibitory neurotransmitters are the amino acids γ-aminobutyric acid (GABA) and glycine, which are predominantly released from intrinsic interneurons. Iontophoresis of GABA and glycine was found to inhibit activity in intracellularly and extracellularly recorded dorsal horn cells and motoneurons in the anaesthestised cat in the 1950s and 1960s (Curtis 1959, Curtis et al. 1967, 1968; Werman et al. 1967). Subsequent experiments confirmed these findings in the monkey (Willcockson et al. 1984), in adult rat spinal cord preparations (Zieglgansberger and Sutor 1983; Long et al. 1989), and in dissociated rat dorsal horn neurons (Murase et al. 1989). Conversely, the GABA and glycine ionotropic receptor antagonists bicuculline and strychnine produce disinhibition of rat and cat dorsal horn neurons and motoneurons (Sivilotti and Woolf 1994; Sorkin and Puig 1996;

Reeve et al. 1998), and block inhibitory postsynaptic currents recorded by patch clamping in rat spinal cord slices (Yoshimura and Nishi 1995) and *in vivo* (Narikawa et al. 2000).

1.3.1 GABA

Approximately 20% of neurons in the adult cord are positive for GABA (Schaffner et al. 1993), which is synthesised in the cord by glutamic acid decarboxylase (GAD). GABA acts on both ligand-gated anion channels [the chloride (Cl^-) and bicarbonate (HCO_3^-) permeable $GABA_A$ receptors ($GABA_ARs$); $GABA_C$ receptors] and metabotropic G-protein coupled receptors ($GABA_BRs$).

1.3.1.1 $GABA_ARs$

The $GABA_AR$ has a heteropentameric quaternary structure and a molecular weight of approximately 230 kDA (Mehta and Ticku 1999). There are seven known receptor subunit types (α, β, γ, δ, ε, η, and θ) and multiple isoforms exist within the α, β, and γ subunits, leading potentially to a very large heterogeneity in $GABA_AR$ subunit composition. However, the predominant composition in adult spinal cord follows the pattern $(\alpha x)_2 (\beta 2 \text{ or } \beta 3)_2 (\gamma 2)_1$ (Ma et al. 1993).

Immunostaining for the β2 and β3 subunits demonstrated reactivity throughout the dorsal horn, the most intense staining in laminae II and III (Alvarez et al. 1996). Other studies using antibodies against the γ2 subunit (Sur et al. 1995) and various α subunits (Bohlhalter et al. 1996) similarly show widespread staining for these subunits, with the latter again noting a particularly abundant expression in superficial laminae. The functional activity of these $GABA_ARs$ was demonstrated by intrathecal injection of rats with with the $GABA_AR$ antagonist picrotoxin, which resulted in an increase in the number of neurons displaying Fos-like immunoreactivity (an immediate-early gene marker of activity) throughout the dorsal

horn, peaking in laminae II and V, suggesting that cells in these areas are tonically inhibited by GABA (Cronin et al. 2004).

In the postsynaptic membrane, $GABA_ARs$ mediate fast inhibitory neurotransmission by allowing Cl^- to enter and hyperpolarise the postsynaptic cell. These transient or phasic inhibitory currents are usually on the timescale of 10 - 100 ms (Semyanov et al. 2004). $GABA_ARs$ can also be found extrasynaptically in many areas of the CNS, where they mediate a slow, tonic inhibitory current (Kullmann et al. 2005). These extrasynaptic receptors usually contain the δ subunit, which is absent from the spinal cord, but there is some evidence for an inhibitory current mediated by extrasynaptic $GABA_ARs$ in superficial dorsal horn at least (Chery and De Koninck 1999).

$GABA_ARs$ are also present on the presynaptic terminal (Labrakakis et al. 2003) where they mediate primary afferent depolarization (PAD). This is a form of presynaptic inhibition that relies on a high concentration of intracellular Cl^- within the nerve terminal, which is maintained by expression of the co-transporter NKCC1 (Sung et al. 2000). In this situation, activation of presynaptic $GABA_ARs$ results in an outward anion flux, and consequent depolarisation of the nerve terminal. This depolarisation and increased membrane conductance shunts the magnitude of invading action potentials and decreases excitatory neurotransmitter release (Rudomin and Schmidt 1999).

1.3.1.2 $GABA_BRs$

Slow synaptic inhibition is mediated by $GABA_BRs$, metabotropic receptors coupled to heterotrimeric G proteins. In contrast to $GABA_ARs$, there are only three known $GABA_BR$ subunits, $GABA_BR$ 1a, 1b and 2 (Bettler et al. 2004). In situ and immunocytochemical studies have shown that all three subunits are present throughout the dorsal horn and that $GABA_BRs$ are located both on primary afferent terminals and on postsynaptic cell bodies (Towers et al. 2000).

$GABA_BRs$ are functionally coupled via G-proteins to potassium (K^+) and calcium (Ca^{2+}) ionotropic channels. Postsynaptically, the opening of K^+ channels after $GABA_BR$ activation causes delayed and long lasting inhibitory potentials. Presynaptically, $GABA_BR$ activation leads to a decrease in membrane Ca^{2+} conductance and a subsequent reduction in neurotransmitter release. The presynaptic receptors sometimes act as autoreceptors for GABA, and hence can form negative feedback loops (Kerr and Ong 1995).

1.3.1.3 $GABA_CRs$

Like the $GABA_AR$, $GABA_CRs$ are ionotropic pentamers, but are composed of ρ subunits (ρ1-5 currently identified) and, unlike $GABA_ARs$, are not anatagonised by bicuculline or activated by baclofen (Chebib and Johnston 2000). $GABA_CRs$ were initially described in the retina, and ρ subunits have subsequently been localised in other CNS regions, such as the thalamus, the hippocampus and the spinal cord (Enz et al. 1995; Rozzo et al. 2002). Within the adult rat spinal cord, ρ subunit protein has been observed in all laminae throughout both dorsal and ventral horns, and on interneurons as well as motoneurons (Rozzo et al. 2002). However, the physiological effect of these receptors is still unclear, as a combination of $GABA_AR$ and glycine receptor antagonist is sufficient to block evoked inhibitory currents in the superficial dorsal horn (Moore et al. 2002).

1.3.2 Glycine

Levels of glycine are highest in the ventral horn, in accordance with its essential role in spinal reflexes (Aprison and Werman 1965), but it is also present throughout the dorsal horn and is critical in the processing of sensory signals. At present only one glycine receptor (GlyR) is known.

1.3.2.1 Glycine receptors

The glycine receptor (GlyR) has a molecular weight of approximately 260 kDa and a pentameric structure composed of the subunits α1-4 and β, with the most likely stoichiometry in the adult being $(\alpha 1)_3$ $(\beta)_2$ (Kuhse et al. 1993). As with $GABA_A$Rs, GlyRs are ligand-gated anion channels and mediate fast synaptic inhibition via influx of Cl^-. GlyRs are widespread within the spinal cord, and have a similar distribution to glycine itself. In parallel with the picrotoxin experiments (see section 1.2.1.1), Cronin et al. (2004) intrathecally injected rats with the GlyR antagonist strychnine and again examined the expression of Fos in the dorsal horn. They observed increased Fos-like immunoreactivity in laminae III-VI only, indicating a particular role in tonic inhibition for GlyRs in these deeper laminae.

1.3.2.2 Co-transmission by GABA and glycine?

Many interneurons are both GABAergic and glycinergic (Todd et al. 1996), and GABA and glycine are known to be co-released from individual vesicles at some synapses (Jonas et al. 1998; Chery and De Koninck 1999). However, in superficial adult dorsal horn, it appears that fast inhibitory postsynaptic currents (IPSCs) are only ever mediated by either $GABA_A$Rs or GlyRs, never both. For example, in lamina I it has been reported that IPSCs are entirely GlyR-mediated, despite known co-release of GABA and glycine at synapses in this lamina [(Chery and De Koninck 1999), but c.f. (Puskar et al. 2001)]. Similarly, in lamina II, both $GABA_A$R- and GlyR-mediated IPSCs were observed, in an approximately even distribution, but the postsynaptic currents were never mixed at a single synapse, arguing for postsynaptic specificity in $GABA_A$R and GlyR synaptic expression (Chery and De Koninck 1999).

1.4 Descending modulation of adult dorsal horn circuits

Supraspinal modulation of spinal cord circuits was reported as early as 1915 by Sherrington, who described an increase in the flexion reflex after spinalisation in the decerebrate, spinalised cat (Sherrington and Sowton 1915). Later experiments showed that reversible blockade of descending pathways by cooling the cord at thoracic levels caused a temporary increase in spontaneous activity, receptive field size and neuronal excitability of single dorsal horn cells, also in the decerebrate cat (Wall 1967). Both of these results provided strong evidence for a tonic inhibitory influence on the spinal cord from higher centres. The strength of these descending inhibitory controls was revealed in a startling paper by Reynolds (1969) in which electrical stimulation of the periaqueductal grey (PAG) in rats produced antinociception to such a degree that surgery could be carried out without anaesthesia. The potential for pain relief in humans by harnessing the same pathways was obvious, and indeed stimulation of the PAG in humans does produce analgesia (Hosobuchi et al. 1977; Richardson and Akil 1977). The emphasis of research in this field remained largely on descending inhibition for some years, but supraspinal modulation of dorsal horn circuitry is now known to be more complex, and involves descending facilitatory or pronociceptive pathways in addition to the antinociceptive inhibitory pathways originally described.

The complexity of descending modulation is due in part to the multiplicity of brainstem regions (and higher centres) which project directly or indirectly to the spinal cord, and also in part to the various different transmitter systems which are known to be involved. These different transmitters can to some extent be classified as mediating either descending inhibition or descending facilitation; however, in several cases a single transmitter may produce bidirectional modulation of spinal nociceptive processing owing to the presence in the cord of different receptor subtypes with opposing actions on neuronal excitability. Moreover, the end target of the descending terminals will have a crucial impact on the direction of modulation. Descending pathways can terminate on primary afferent terminals, excitatory interneurons, inhibitory interneurons, projection neurons, or on the terminals of

other descending pathways, some of which (for example, inhibitory interneurons) would serve to invert the original modulatory effect. Furthermore, many of the descending systems elucidated in the past 30 years have been found to interact with each other at spinal or supraspinal levels, or both, creating yet another level of complexity. Despite this, several of the major anatomical pathways and transmitter systems involved in descending modulation of the dorsal horn have been well characterised and will be briefly described below (see Millan 2002 for a comprehensive review).

1.4.1 Anatomy of the major descending pathways

The major monosynaptic inputs to the dorsal horn arise from brainstem nuclei, foremost of which are the rostroventral medulla [RVM; including the nucleus raphe magnus (NRM)], the parabrachial nucleus, the dorsal reticular nucleus (DRN), and the A7 nucleus of the dorsolateral pontine tegmentum (DLPT). These send axons to the dorsal and ventral horns via the dorsolateral funiculus (DLF) and the ventrolateral funiculus (VLF). It has been proposed that the DLF mediates descending inhibition and the VLF descending facilitation (Zhuo and Gebhart 1997; Watkins et al. 1998), but DLF stimulation has been reported to excite cells in the dorsal horn (McMahon and Wall 1988) and the serotonergic descending facilitation associated with neuropathic pain is reduced after DLF transection (Ossipov et al. 2000), thus a sharp categorisation in this way may not be accurate. The projections from the parabrachial nucleus terminate preferentially in the superficial dorsal horn (Yoshida et al. 1997) whereas those from the RVM, NRM, DRN and A7 nucleus project to both superficial and deep laminae. Within the dorsal horn, WDR cells are an important target for descending modulation and studies have shown that activation or block of descending pathways can depress the sensitivity of WDR cells to noxious stimuli relative to innocuous stimuli, as well as altering the RF area (Wall 1967; Willis 1988; Fields et al. 1977; Hudson et al. 2000).

As mentioned above, the periaqueductal grey (PAG) has long been known to play a crucial role in descending inhibition. However, it has few direct projections to the dorsal horn and

instead has strong links to the RVM and the DLPT and drives monoaminergic descending inhibition from these areas. The DLPT receives a substantial projection from the RVM and so can also be driven by an indirect PAG – RVM – DLPT pathway. The PAG – brainstem pathways are themselves under tonic GABAergic inhibition which can be relieved by administration of opioids in the PAG, thereby releasing descending inhibition. Opioids play a major role in the control of descending modulation at supraspinal levels, especially in the RVM and PAG, and this is thought to be a key mechanism in opioid-induced analgesia.

1.4.2 Major neurotransmitter and receptor systems

There are two major groups of transmitters which are involved in mediating the effects of supraspinal centres on the dorsal horn: monoamines (noradrenalin, serotonin and dopamine), which are the neurotransmitters predominantly involved in descending modulation, and non-monoamines (a diverse group including GABA, glycine, acetylcholine and cannabinoids). Monoamines are not intrinsic to the spinal cord and are released into the dorsal horn only from the terminals of descending projections. This makes their contribution to descending modulation easier to assess than for neurotransmitters such as GABA which are endogenous to the spinal cord as well as released from descending fibres. Although the descending systems are presented here as distinct pathways, there is a large degree of interaction between them, both segmentally and at supraspinal levels.

1.4.2.1 Noradrenergic pathways

Noradrenergic (NA) innervation of the spinal cord is mainly derived from the pontine A7 nucleus, which projects to laminae I, II, IV, V and X. Electrical stimulation of the A7 nucleus and spinal application of NA both elicit robust antinociceptive behaviour (Reddy and Yaksh 1980; Yeomans et al. 1992) and inhibit dorsal horn neurons in the rat (Howe and Zieglgansberger 1987) and cat (Headley et al. 1978). The α_2 adrenoceptor (α_2-AR) is a G-protein coupled receptor (GPCR) which mediates the inhibitory role of NA in the dorsal horn

by enhancing potassium currents and suppressing calcium currents, both of which serve to decrease neuronal excitability. Agonists at the α_2-AR such as clonidine and dexmedetomidine are very effective in reducing evoked neuronal responses in the dorsal horn, and are used clinically in the treatment of pain (Sullivan et al. 1992; Eisenach et al 1996). The major site of action of NA in the dorsal horn is thought to be postsynaptic, on interneurons and projection neurons, but there may also be an effect on primary afferent terminals (Millan 1997).

1.4.2.2 Serotonergic pathways

The major supraspinal source of serotonin or 5-hydroxytryptamine (5-HT) is the RVM, and the NRM in particular. It is worth noting, however, that the NRM also contains other, non-serotonergic, neurons. Serotonergic terminals are present throughout the dorsal horn, and are particularly dense in laminae I, II, IV, V and VI. Some terminals directly contact projection neurons, others synapse with interneurons. In the original literature, the RVM serotonergic descending pathway was thought to mediate descending inhibition; however, it is now known to be bidirectional and has been observed to have both inhibitory and facilitatory effects on resting and evoked activity, superficial and deep cells, and NS and WDR cells (Millan 2002).

This multiplicity of effects is due at least in part to the diversity of 5-HT receptors. The majority of 5-HT receptors are GPCRs which act on the potassium and calcium currents to increase or decrease neuronal excitability. 5-HT_{1A}Rs are highly expressed in the dorsal horn, particularly in the superficial laminae, and act to decrease neuronal excitability. However, it has been suggested that a substantial proportion of 5-HT_{1A}Rs may be located on GABAergic interneurons, so that their activation could result in disinhibition and a pronociceptive effect (Millan et al. 1996). Other 5-HT receptors present in the dorsal horn which act to decrease neuronal excitability include 5-HT_{1B}Rs, 5-HT_{1D}Rs, and 5-HT_{1F}Rs, which are all thought to have mostly antinociceptive actions. Conversely, 5-HT_{2A}Rs, 5-HT_{2B}Rs, 5-HT_{2C}Rs, 5-HT_4Rs, 5-HT_6Rs, and 5-HT_7Rs all have a facilitatory effect on neuronal excitability and are likely to

have a pronociceptive effect due to their probable location on primary afferent fibres as well as projection cells and interneurons.

Distinct from all the other known 5-HT receptors are the ionotropic 5-HT_3Rs. These are pentameric, ligand-gated, cation-permeable ion channels, which therefore have an excitatory effect when activated. Expression of 5-HT_3Rs is concentrated in superficial laminae, particularly lamina I, where they are thought to be localised mainly on C fibre terminals. They have a well-characterised pronociceptive role and are thought to be the substrate for the descending branch of the lamina I - supraspinal - dorsal horn loop which can set the excitability level of the dorsal horn. This facilitatory pathway has been suggested to play a crucial role in the hypersensitivity observed following nerve injury (Suzuki et al. 2004a, 2004b).

1.4.2.3 Dopaminergic pathways

Cerebral structures, such as the A11 nucleus in the hypothalamus, are the main source of dopaminergic (DA) projections to the spinal cord, and terminals are seen throughout the dorsal horn. D_1 and D_2 receptors are both found in the dorsal horn, though D_2 are the most prominent. D_2 receptors are expressed mainly in the superficial laminae and lamina X and act to decrease calcium currents and increase potassium currents such that neuronal activity is inhibited. Stimulation of the A11 nucleus and spinal application of D_2 agonists elicit antinociceptive behaviour and depress the responses of neurons to noxious stimuli in the cat and the rat (Jensen and Yaksh, 1984; Fleetwood-Walker et al. 1988).

1.4.2.4 Other transmitters

There is a multitude of non-monoaminergic transmitters which seem to be involved in descending modulation, some of which are present only in descending fibres (for example, histamine, vasopressin and oxytocin) and others that are also produced by intrinsic

interneurons or primary afferents. GABA and glycine are contained in a proportion of the descending serotonergic RVM neurons that terminate in the superficial laminae (Maxwell et al. 1996) and GABA is also colocalised with NA in descending noradrenergic fibres (Iijima et al. 1992). Recent experiments combined RVM stimulation with patch-clamp recordings in anaesthetised rats *in vivo* to demonstrate that RVM stimulation produced monosynaptic bicuculline- and strychnine-sensitive IPSCs in substantia gelatinosa neurons (Kato et al. 2006). This is clear evidence for descending inhibition mediated directly by GABA or glycine, or both.

1.4.3 Physiology of the RVM: 'on' and 'off' cells

RVM neurons have been the subject of intense physiological study owing to their distinct and behaviourally relevant firing patterns during the tail-flick withdrawal reflex from noxious heat. In the lightly anaesthetised rat, 'on' cells are generally quiescent but have a burst of activity immediately prior to the tail-flick, whereas 'off' cells are spontaneously active but have a clear pause in firing just before the withdrawal reflex. 'Neutral' cells show no alteration in activity during the noxious stimulation or the reflex response (Fields et al. 1983). These characteristics led to the hypothesis that off cells mediate tonic descending inhibition from the RVM and on cells mediate descending facilitation. However, somewhat surprisingly, it was found that neither on nor off cells are serotonergic (Potrebic et al. 1994), indicating that the RVM can modulate spinal processing through several distinct serotonergic and non-serotonergic pathways. It is possible that the off cell descending pathway may correspond to the GABAergic or glycinergic tract reported by Kato et al. (2006).

A simple circuit has been proposed whereby on cells inhibit off cells via GABAergic synapses or interneurons (Fields et al. 1991). On cells are directly inhibited by morphine, which then indirectly activates the off cells via disinhibition (Heinricher et al. 1992). It has been reported that some GABAergic interneurons in the RVM express the μ opioid receptor as well and so would also be inhibited by morphine, thereby increasing the disinhibition of off cells

(Kalyuzhny and Wessendorf 1998). The antinociceptive effects of morphine in the RVM are hence likely to be two-fold: inhibition of the pronociceptive on cells, and activation of the antinociceptive off cells.

There are difficulties associated with this physiological characterisation, chief of which is that it is difficult at present to identify on and off cells morphologically, neurochemically, or in slice electrophysiology. The on and off cell types are defined by the relation of their firing patterns to the tail-flick reflex, and so require an *in vivo* preparation. They can be distinguished somewhat by responsiveness, or lack thereof, to morphine but other subsets of cells are also likely to express the μ opioid receptor. Differential expression of other types of opioid receptor, such as the κ opioid receptor, may help with discrimination in the future (Winkler et al. 2006). Other classifications of RVM cells have been proposed from different preparations (for example the primary and secondary cells recorded *in vitro*) but work to relate these to on, off, and neutral cells is still ongoing.

1.5 Lack of inhibition in the neonatal dorsal horn?

Studies on the development of spinal reflexes in animals and humans and on the properties of dorsal horn neurons at different ages indicate that substantial changes occur in dorsal horn circuitry over the postnatal period. With development, cutaneous reflex thresholds increase and withdrawal reflexes become more precise and appropriately directed. At the neuronal level, cutaneous mechanical thresholds also increase, cutaneous receptive fields decrease in size, and cells no longer sensitise to innocuous stimulation. These findings are discussed in more detail below.

1.5.1 Spinal reflexes are more excitable in the newborn

In human infants, the threshold cutaneous force necessary to produce a withdrawal is low in premature infants and increases with postconceptional age (PCA) and, for infants younger

than 35 weeks PCA, repeated application of an innocuous stimulus causes sensitisation of the reflex, observed as a subsequent lowering of the threshold (Andrews and Fitzgerald 1994; Andrews et al. 2002). Similarly, reflex thresholds in rat pups also increase postnatally, and the duration of the reflex response is greatly prolonged in neonatal animals compared with adults. This can be observed both behaviourally (Fitzgerald et al. 1988) and by recording the electromyograph (EMG) response in lightly anaesthetised animals (Walker et al. 2005). Lower thresholds at younger ages could be accounted for by decreased skin thickness, more sensitive primary afferents, or differences in ventral horn processing, as well as by differences in the dorsal horn. However, it has been demonstrated that the thresholds for activating cutaneous mechanosensitive primary afferent fibres *in vivo* and in an *in vitro* skin and nerve preparation are similar at all ages (Fitzgerald 1987). Moreover, the stretch reflex, which uses the same set of ventral horn motoneurons as those involved in hind-limb withdrawal, is normal at birth (Kudo and Yamada 1985). This indicates that the lower reflex thresholds in the neonate are most likely due to differences in central processing in the dorsal horn.

1.5.2 Reflexes are imprecise and poorly directed

Cutaneous reflex receptive fields for individual muscles involved in hind-paw withdrawal have been mapped quantitatively using EMG measurements. In the adult rat, each receptive field is spatially restricted to one small region of the hind paw surface. Moreover, the receptive field for each muscle corresponds to the region of the hind paw which would be raised if that muscle contracted; in other words, the reflex receptive fields are tightly and appropriately tuned for each muscle. When the same reflex receptive fields are measured on the third postnatal day (P3), they are more diffuse than in the adult, and have several spatially separated "hotspot" areas of greater activation which are often located in plantar regions inappropriate for the muscle (Holmberg and Schouenborg 1996).

When reflexes are measured behaviourally they appear poorly directed in young animals, which is consistent with the disorganised reflex receptive fields. For example, in mature

animals, the response to noxious heat applied discretely to one side of the tail is a flick of the tail away from the stimulus. However, when this experiment was repeated in P10 pups, ~65% of the tail flicks were in the opposite direction, towards the stimulus. The percentage of incorrect tail-flicks decreased with age, reaching <10% by P21 (Waldenstrom et al. 2003). Subsequent experiments demonstrated that intact low-threshold or tactile input is necessary for the postnatal tuning of this nociceptive reflex (Waldenstrom et al. 2003). Withdrawal reflexes in the human infant also undergo a substantial degree of postnatal tuning. Application of a mechanical stimulus (nylon filaments of graded stiffness known as von Frey hairs) to the abdomen provoked reflex involvement of both lower limbs in ~70% of preterm subjects (32 weeks PCA). As the age of the subjects increased, the reflex became increasingly restricted, first to unilateral lower limb involvement (by approximately 38 weeks PCA), and then to the abdominal muscles only (by 95 weeks PCA) (Andrews et al. 2002).

1.5.3 Dorsal horn cell properties

In an anaesthetised *in vivo* preparation, dorsal horn cells recorded from neonatal (P3) rats had lower mechanical thresholds than those from P21 rats, and when repetitive low-threshold stimulation was applied to the skin, sensitisation of the afterdischarge spiking was commonly observed at P3 but not at P21 (Fitzgerald 1985; Jennings and Fitzgerald 1998; Torsney and Fitzgerald 2002). Also, it has been well documented that cutaneous receptive fields are larger relative to the size of the paw in P3 rats than in P21 and adult rats (Fitzgerald 1985; Torsney and Fitzgerald 2002). These properties of second-order dorsal horn neurons during the early postnatal period mirror to some extent the properties of the developing withdrawal reflex and, similarly, cannot be accounted for merely by changes in the skin or primary afferent fibres. A lack of efficacy of inhibitory interneurons, however, could underlie both the increased excitability and enlarged receptive fields of neonatal dorsal horn cells (see figure 1.2).

1.6 Postnatal development of the dorsal horn

1.6.1 Developmental regulation of GABA and glycine

The importance of GABAergic transmission in the neonatal spinal cord is shown by the high proportion of GABA-positive cells: 50% at birth declining to ~20% in the adult (Schaffner et al. 1993). There are few postnatal changes in the $GABA_AR$ subunit composition, except for the γ2 subunit, which is more highly expressed during the first postnatal week (Ma et al. 1993; Pattinson and Fitzgerald 2004).

In contrast to $GABA_ARs$, the subunit composition of spinal GlyRs does undergo substantial postnatal regulation: the α2 subunit is highly expressed at birth and is gradually replaced by the α1 subunit over the first three postnatal weeks (Watanabe and Akagi 1995). This could be functionally important because the mean open time for α2-containing GlyRs is approximately sixty times longer than for α1-containing GlyRs, producing longer current decay times for neonatal GlyRs (Takahashi et al. 1992).

1.6.2 Developmental neuroanatomy and neurophysiology

1.6.2.1 Patterns of primary afferent innervation

The low-threshold, large myelinated Aβ fibres are the first primary afferents to enter the spinal cord, at approximately embryonic day (E) 15, and initially a proportion terminates in superficial laminae as well as in laminae III - V (Torsney et al. 2000; Beggs et al. 2002). Trk-A positive C fibres enter the cord a few days later at E18-E20 and terminate in their final positions in laminae I and II, and isolectin B4 positive (IB4+) C fibres terminals appear later still, from P5 onwards, when they terminate specifically in lamina II (also known as the substantial gelatinosa, or SG) (Pignatelli et al. 1989; Jackman and Fitzgerald 2000; Benn et al. 2001). At birth both Aβ and C fibre terminals innervate the superficial laminae, and there is a gradual NMDA receptor-dependent withdrawal of Aβ fibre terminals over the first few

postnatal weeks until they are restricted to the adult termination pattern in laminae III - V (Beggs et al. 2002). Studies of Fos expression following low-threshold cutaneous stimulation are consistent with this: low-threshold stimulation produces Fos activation in superficial laminae at P3 but not at P21 (Jennings and Fitzgerald 1996). In addition, patch-clamp recordings from SG neurons showed a much greater incidence of monosynaptic Aβ-evoked inputs in spinal cord slices from P21 rats than from ~P60 rats (51% of cells at P21, compared with 9% of cells at P60) (Nakatsuka et al. 2000) again indicating a developmental withdrawal of the direct Aβ-fibre input to the superficial layers.

1.6.2.2 Postnatal development of C fibre inputs

In the adult, dorsal horn cells with a C-fibre input show a burst of activity ~ 100 - 300 ms after peripheral cutaneous stimulation that can be clearly distinguished from A-fibre-evoked activity, which has a much shorter latency of ~ 5 - 50 ms. The C-fibre inputs have such a long latency owing to their small diameter and lack of myelination. However, extracellular recordings *in vivo* have demonstrated that peripheral stimulation at C-fibre intensities does not result in these long-latency spike responses in P3 or P6 pups, (Jennings and Fitzgerald 1998), despite the known maturity of the peripheral afferents at this age and the presence of their terminals in the dorsal horn. The classical C-fibre response begins to appear only around P10, indicating a late maturation of the synapses from the primary afferent terminals to the dorsal horn neurons. When C-fibre terminals were directly activated by applying capsaicin to spinal cord slices *in vitro*, though, a clear increase in the frequency of miniature excitatory postsynaptic currents (mEPSCs) was observed in postsynaptic dorsal horn cells even at P0 (Baccei et al. 2003), indicating that the synapses are indeed functional from an early age but merely remain sub-threshold for postsynaptic spike generation *in vivo*. The capsaicin-induced increase in mEPSC frequency became much stronger between P5 and P10, supporting the hypothesis that although some functional synapses are present at birth, the vast majority are formed between P5 and P10. Moreover, the glutamate release induced by capsaicin application at the young ages was asynchronous, so it is possible that synchronous release of

sufficient vesicles to evoke action potentials in the postsynaptic cell does not occur in response to natural stimulation in younger animals, explaining the lack of spike activity recorded *in vivo* until P10 (Baccei et al. 2003).

1.6.2.3 Postnatal development of intrinsic synaptic transmission

In addition to the above-mentioned absent or weak C-fibre evoked spike activity *in vivo*, the efficacy of synaptic transmission within the dorsal horn in general appears to be low at birth and increases over the immediate postnatal period: spontaneous EPSCs (sEPSCs; a general indicator of activity in the network) in superficial dorsal horn are upregulated during the first ten postnatal days (Baccei et al. 2003). Similar results have been found regarding the postnatal development of IPSCs as the frequencies of miniature and spontaneous IPSCs both increase greatly over the first two weeks (Baccei and Fitzgerald 2004).

Furthermore, there is a developmental shift in the balance of neurotransmitters mediating IPSCs within the superficial dorsal horn. The $GABA_AR$ antagonist bicuculline blocked almost all sIPSCs during the first postnatal week whereas the GlyR antagonist strychnine had no significant effect, suggesting a predominant role for $GABA_ARs$ in fast inhibitory transmission during this time (Baccei and Fitzgerald 2004). By P14, GlyR-mediated IPSCs could be observed alongside $GABA_AR$-mediated IPSCs. Exogenous application of glycine produced clear inward currents in cells at all ages, showing that the lack of GlyR-mediated IPSCs during the first week is not due to an absence of functional GlyRs. Instead, it is more likely to be because the receptors are not yet clustered appropriately in the postsynaptic density, and so are not activated by synaptically released glycine. This hypothesis is supported by immunohistochemical and Western blot analysis of the expression of gephyrin, a scaffolding protein known to be necessary for clustering of GlyRs at synaptic clefts (Kirsch et al. 1993; Feng et al. 1998). The upregulation of gephyrin in the postnatal dorsal horn closely matches the time course of development of GlyR-mediated IPSCs (E. Harrop and M. Fitzgerald, unpublished observations). Interestingly, the postsynaptic specificity for either

$GABA_AR$- or GlyR-mediated IPSCs that has been reported in adult superficial dorsal horn (Chery and De Koninck 1999), and see section 1.3.2.2 above) emerges only in adulthood. In slices from animals younger than P23, mixed $GABA_AR$-GlyR IPSCs were observed alongside $GABA_AR$-only and GlyR-only IPSCs (Keller et al. 2001).

Developmental changes also occur in the kinetics of the $GABA_AR$-mediated mIPSCs, which decay at a slower rate in neonatal dorsal horn cells than in mature cells (Keller et al. 2004). The slow decay in immature slices is thought to be due to tonic endogenous production of 5alpha-reduced neurosteroids during development, which ceases in adulthood. This longer decay time for $GABA_AR$-mediated IPSCs could have important implications for signal processing in the immature spinal cord because not only does more total charge pass across the membrane, but the time period for integration with other synaptic inputs is also increased.

1.6.2.4 Postnatal development of descending modulation

The descending modulation which plays a crucial role in spinal cord processing in the adult (section 1.4) appears to mature over the first three postnatal weeks in the rat (see also chapter 4). Electrical stimulation of descending tracts inhibits evoked firing in dorsal horn cells only after P12, and does not reach adult levels until the third postnatal week (Fitzgerald and Koltzenburg 1986). Similarly diffuse noxious inhibitory controls (DNIC), a form of heterotopic inhibition dependent on descending tracts, are not observed prior to P21 (Boucher et al. 1998), and stimulation of the midbrain elicits antinociception only after a similar age (van Praag and Frenk 1991). Furthermore, the biphasic shape of the behavioural response to formalin, which has been attributed to descending influences, is not observed before P15 (Guy and Abbott 1992). Recent work suggests that the developmental differences observed may not be due simply to a lack of descending inhibition in the neonate but may be due to a shift in the excitatory – inhibitory balance of descending modulation over the postnatal period (Hathway et al. 2006).

1.7 Aims of the thesis

It is clear from cellular and molecular studies that the inhibitory system in the dorsal horn undergoes substantial developmental changes during the postnatal period. This led to the hypothesis that the maturation of inhibitory circuitry is responsible for the changing properties of sensory and nociceptive reflexes and behaviour in postnatal animals. The overall aim of this thesis is to test this hypothesis in an *in vivo* preparation and assess the functional significance of postnatal changes in inhibition in the context of the whole animal. To do this I have studied the maturation of segmental and descending inhibitory processing in the rat dorsal horn using electrophysiological methods.

In chapter 2, I test the importance of the known cellular and molecular changes in inhibitory transmission and signalling in the dorsal horn, at the systems level. Single dorsal horn cells are recorded at two postnatal ages over a prolonged period in the presence and absence of a $GABA_AR$ antagonist to test the role of GABAergic signalling on dorsal horn cell properties *in vivo*.

Chapter 3 examines the role of descending activity from supraspinal centres upon dorsal horn activity at different ages, to test how individual dorsal horn cells are influenced by descending activity during development and how this may affect spinal inhibitory processing in young animals.

In chapter 4, I examine the network-level organisation of inhibitory processing in the dorsal horn by mapping the spatial pattern of inhibitory receptive fields at different ages. The results are used to propose a model for the developmental pattern of inhibitory receptive field organisation and the role of such fields in higher-level sensory processing.

Chapter 2

Functional GABAergic inhibition in the neonatal dorsal horn

2.1 Introduction

It has recently been discovered, first in the hippocampus and subsequently in several other regions of the central nervous system, that ionotropic receptors usually associated with fast synaptic inhibition, such as the $GABA_AR$, under some conditions mediate excitation (Ben Ari 2002). Moreover, this is most commonly observed as a developmental phenomenon, occurring during approximately the first two postnatal weeks. It is likely that the immature spinal cord also undergoes a period of GABA excitation during development, and it is possible that this underlies the more excitable responses seen in reflex behaviour, EMG experiments and single-unit recordings in the dorsal horn during the early postnatal period.

2.1.1 The chloride gradient and KCl co-transporters

Fast synaptic inhibition is driven by a transmembrane concentration gradient for chloride ions. A high extracellular and low intracellular chloride concentration ($[Cl^-]$) result in a chloride reversal potential (E_{Cl}) more negative than the resting membrane potential. Thus, when Cl^--permeable $GABA_ARs$ and GlyRs are opened, there is an influx of chloride ions and consequent hyperpolarisation of the membrane (see figure 2.1). During the developmental periods of GABA excitation, the chloride concentration gradient is reversed and E_{Cl} shifts to become more positive than the resting membrane potential. Channel opening in this situation causes an efflux of chloride ions and depolarisation rather than hyperpolarisation of the membrane. If the shift in E_{Cl} is great enough, channel opening may even lead to frank excitation.

The appropriate intracellular $[Cl^-]$ is maintained by the co-transporters KCC2 and NKCC1. These are both members of a large family of cation – chloride co-transporters including KCC1 to 4, NCC, and NKCC1 to 2, which have many functions in the body, including regulation of cell volume. NKCC1 is present throughout the CNS, in glial and endothelial cells as well as in neurons, and is widely distributed in other tissues (Russell 2000). In neurons, NKCC1

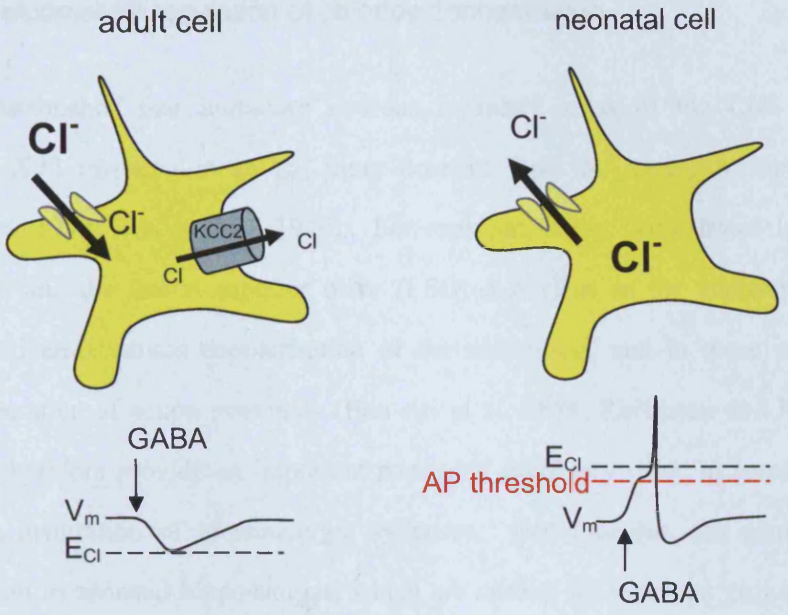


Figure 2.1 Diagram of the developmental shift in intracellular chloride concentration
In adult neurons, a low intracellular chloride concentration is maintained by the K^+ Cl^- cotransporter KCC2, ensuring that activation of $GABA_A$ receptors causes influx of Cl- ions and hyperpolarisation of the membrane (left). In some neonatal neurons, a reversed Cl^- concentration gradient has been observed. In this situation (right), activation of $GABA_A$ receptors causes efflux of Cl^- and membrane depolarisation, which may result in firing of action potentials. [V_m - resting membrane potential; E_{Cl} - chloride reversal potential; AP - action potential]

causes intracellular accumulation of Cl^- (Sun and Murali 1999), which is necessary in some specialised situations, for example presynaptic inhibition at primary afferent terminals and during development (Price et al. 2005).

KCC2 is neuron–specific and actively extrudes chloride from neurons, maintaining the low intracellular [Cl^-] necessary for fast hyperpolarising inhibition (Rivera et al. 1999). KCC2 is cycled in the membrane over a time scale of just minutes (Rivera et al. 2004), enabling rapid up or downregulation in some circumstances, and it has recently been suggested that Ca^{2+}-dependent modulation of KCC2 over an even shorter time scale may play a role in short-term plasticity at inhibitory synapses (Woodin et al. 2003).

2.1.2 Developmental regulation of chloride concentration

It is well established that immature neurons in many areas of the CNS have a high intracellular [Cl^-] resulting in an E_{Cl} more positive than the resting membrane potential (Ehrlich et al. 1999; Rivera et al. 1999). For example, it has been shown in the neonatal hippocampus and the lateral superior olive (LSO; a nucleus in the auditory system) that $GABA_AR$ activation causes depolarisation of the membrane, and in these areas can even provoke generation of action potentials (Ben Ari et al. 1989; Kullmann and Kandler 2001). GABA can therefore provide an important source of excitatory drive in some CNS regions prior to the maturation of glutamatergic synapses. For example, the giant depolarising potentials seen in neonatal hippocampus, which are critical for neuronal growth and synapse formation, are mediated by GABA (Ben Ari et al. 1989).

The reversed chloride gradient in immature neurons is due to the relative expression of KCC2 and NKCC1 during early development. NKCC1 mRNA expression is high in the rat brain throughout the first two postnatal weeks and during this period is responsible for the intracellular accumulation of Cl^- in immature neurons (Yamada et al. 2004). After postnatal day (P) 14, NKCC1 expression declines and it is only weakly expressed in the adult CNS (Plotkin et al. 1997). KCC2 expression follows an inverse pattern: it is barely detectable in most CNS regions until ~P5 then it is upregulated over the following two weeks (Rivera et al. 1999; Wang et al. 2002), a time course that closely parallels the switch in GABA function from excitatory to inhibitory (Stein et al. 2004). In the hippocampus, several studies have shown that upregulation of KCC2 is both necessary and sufficient for this developmental switch. Antisense blockade of KCC2 expression in mature neurons caused a positive shift in E_{Cl} to resemble that found in immature neurons (Rivera et al. 1999) and, conversely, transfection of immature cells with KCC2 caused a negative shift in E_{Cl}, similar to that in mature neurons (Lee et al. 2005). Experiments with KCC2 knock-out mice are difficult as the mice die soon after birth, probably due to lack of respiration caused by severe motor deficits (Hubner et al. 2001). In contrast, NKCC1 null mice are viable and show relatively few

deficits except in presynaptic inhibition (Sung et al. 2000), confirming that NKCC1 is likely to be less critical for the control of intracellular [Cl^-] than KCC2. The signals which control the upregulation of KCC2 remain to be elucidated, but one hypothesis is that GABAergic excitation is itself a key factor. Blockade of GABA depolarisations with a $GABA_AR$ antagonist prevented normal developmental upregulation of KCC2 (Ganguly et al. 2001). This is an elegant solution as it ensures that GABA excitation is self-limiting and that the switch to an inhibitory role is concurrent with the emergence of glutamatergic excitation; however, other groups have not been able to confirm the hypothesis (Ludwig et al. 2003; Titz et al. 2003).

2.1.2.1 Spinal cord

Activation of $GABA_ARs$ in the embryonic spinal cord causes depolarisation of the postsynaptic membrane similar to that discussed above (Nishimaru et al. 1996; Kulik et al. 2000). However, in the early postnatal spinal cord, the situation is less clear. Motoneurons are inhibited by GABA (exogenous or endogenous) from P0 (Marchetti et al. 2002; Hubner et al. 2001) so the maturation of inhibition in the ventral horn appears to occur at an earlier stage than in other parts of the CNS. In the dorsal horn, immunohistochemistry and Western blots show that expression of KCC2 is very low at P3 but increases significantly by P10 and reaches adult levels by P21 (E. Harrop and M. Fitzgerald, unpublished observations, see figure 2.2). This parallels some of the developmental changes reported in reflex behaviour, EMG experiments and single-unit recordings (see section 1.5) and suggests that GABA excitation in the immature dorsal horn may underlie some of these observations. However, *in vitro* experiments using the perforated-patch technique to preserve physiological intracellular [Cl^-] showed that exogenous GABA did produce depolarisations in some lamina II neurons (40%) at P0 - P2, but that by P6 - P7 GABA always elicited hyperpolarisation. Moreover, the GABA reversal potential (E_{GABA}) remained above the threshold for generation of action potentials even at the youngest ages, so it is unlikely that GABA provides a strong excitatory drive in the postnatal dorsal horn (Baccei and Fitzgerald 2004).

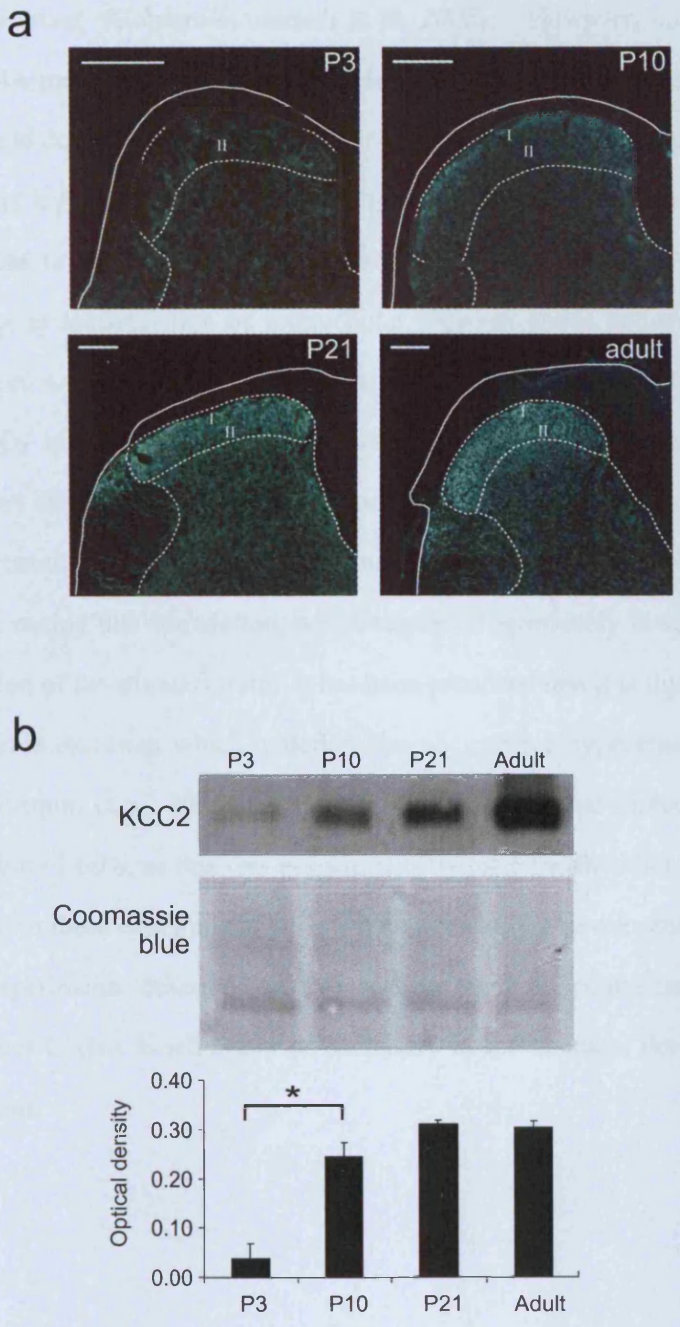


Figure 2.2 (a) Photomicrographs of rat dorsal horn at P3, P10, P21 and adult showing KCC2 immunolabelling. KCC2 is undetectable at postnatal day (P) 3, appears in lamina I at P10, and in laminae I and II at P21 and adult. Scale bars = 100 μm. (b) Western blot analysis demonstrating the postnatal upregulation of KCC2 in the dorsal spinal cord. There is a marked and significant upregulation between P3 and P10 (n = 6 at all ages, * $p<0.05$). Figure used with permission from E. Harrop and M. Fitzgerald (unpublished observations).

A recent study confirmed that E_{GABA} is more negative than the resting membrane potential by P7 in lamina I neurons (Cordero-Erausquin et al. 2005). However, the same study also reported that GABA induced a rise in intracellular Ca^{2+} during the first three postnatal weeks, caused by a rebound depolarisation of the membrane after the initial hyperpolarisation. They concluded that this biphasic response was due to immature chloride transport mechanisms which were unable to maintain sufficient chloride extrusion during prolonged $GABA_AR$ activation, leading to accumulation of intracellular chloride and a collapse of the chloride gradient. Without a prolonged chloride-induced hyperpolarisation, underlying outward movement of HCO_3^- ions resulted in a net secondary depolarisation (Cordero-Erausquin et al. 2005). The authors also investigated whether this effect was of physiological significance by stimulating presynaptically at 20 – 100 Hz, and found that 39% of neurons did show a biphasic response during this stimulation, which tended progressively towards depolarisation through the duration of the stimulus train. It has been proposed that it is this immaturity in the capacity for chloride extrusion which underlies the nociceptive hypersensitivity in neonatal rats (Cordero-Erausquin et al. 2005). However, it is possible that biphasic responses also occur in adult lamina I cells, as this was not specifically tested with either exogenous GABA or trains of stimuli in these experiments, so it is not necessarily a developmental phenomenon. Moreover, the experiments described in this section were all conducted *in vitro*, so the question of whether GABA is inhibitory or excitatory in the neonatal dorsal horn *in vivo* is still open to question.

2.1.3 Aims of the chapter

The aims of the experiments in this chapter were two-fold:

1) To study the postnatal development of dorsal horn cell properties *in vivo* using single unit extracellular electrophysiological recordings, and to provide a set of baseline data for experimental manipulations.

2) To investigate the functional maturation of GABAergic inhibition in intact dorsal horn circuits in the early postnatal rat. The selective $GABA_AR$ antagonist gabazine was applied directly to the spinal cord and the effects of the drug on evoked activity and receptive field size were recorded at P3 and P21. The rationale for this was that increased firing and receptive field areas demonstrate tonic GABAergic inhibition, while little or no change or decreased firing and receptive field areas demonstrate less inhibition, or GABAergic excitation.

2.2 Materials and methods

2.2.1 Animals

Sprague-Dawley rats of both sexes and aged postnatal day 3 (P3) and P21 were obtained from UCL Biological Services. All animals had free access to food and water. P3 rats were housed with their mothers and littermates; P21 rats were housed in single-sex groups of approximately six animals per cage. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986.

2.2.2 *In vivo* electrophysiology

2.2.2.1 Preparation

The animals were anaesthetised with a single intraperitoneal injection of 2.0 – 2.5 g/kg urethane (Sigma, Dorset, UK) in saline. After induction of anaesthesia, as verified by areflexia, a cannula was inserted into the trachea and tied in place with surgical silk. The rat was then secured above a heated blanket (36 °C) with ear and hip bars (Kopf stereotaxic frame). The electrocardiogram (ECG) was monitored via electrodes attached to the fore paws, and heart rate maintained at 300 – 450 beats per minute. Anaesthesia was monitored throughout the duration of the experiment by observing the ECG and behavioural response to a noxious cutaneous pinch. Lack of a withdrawal reflex and no change in the ECG to this stimulation indicated that the depth of anaesthesia was adequate. For the ages used in this experiment, no top-up doses of urethane were ever necessary. A lumbar laminectomy was performed to expose the dorsal surface of the spinal cord at L4 – L5, and the cord was held stable with a rostral vertebral clamp. The dura mater and pia were carefully removed and the surface of the cord bathed in mineral oil (Sigma Aldrich). At the end of the experiment, animals were killed with an overdose of anaesthetic (sodium pentobarbitone i.p).

2.2.2.2 Single-unit extracellular recordings

A reference electrode was placed into the muscle close to the laminectomy. Extracellular recordings were made with 10 μm tip (impedance ~1.5MΩ) glass-coated tungsten microelectrodes (Ainsworks, Welford, Northants), lowered onto the dorsal surface of the cord under microscopic vision, then through the cord in 2 or 10 μm steps by a microdrive (New Brain).

The recording and reference electrodes were connected to a headstage which fed into an amplifier (Neurolog module NL104) where the reference signal was subtracted from the recording signal and the result amplified by 5000. The signal was then band-pass filtered between 1 kHz and 10 kHz with an additional 50 Hz notch filter (module NL125) and the subsequent 'raw data' signal passed simultaneously to an oscilloscope (Tektronix TDS2012), a PowerLab 4SP unit (AD Instruments), and a spike trigger unit (module NL 201). The oscilloscope allowed constant real-time viewing of the raw data signal, and the PowerLab 4SP unit converted the signal from analogue to digital (10 kHz sampling rate) to enable the information to be recorded on a computer (see below). The spike trigger unit generated all-or-none impulses from the raw data signal, with the threshold for generation of impulses being set on the front panel of the unit during each experiment. The impulses from the spike trigger unit were fed into an audio amplifier (module NL120) which was connected up to a speaker so that a clear click could be heard each time an impulse was generated. The information from the spike trigger unit was also sent to a separate channel of the PowerLab 4SP unit.

Single cells were isolated by moving the electrode through the dorsal horn until action potentials of a clearly distinct amplitude (usually ~50 μV) and spike shape could be distinguished reliably from the background noise (usually ~10 μV). Stroking of the plantar skin of the hind paw was used as a search stimulus. The threshold of the spike trigger unit was set such that clicks were only produced by action potentials from the isolated cell. The recording depth from the surface was noted and cells classified as superficial (presumed laminae I and II; depth ≤ 200 μm from the dorsal surface at P3, ≤ 300 μm at P21) or deep

(presumed laminae III, IV, V, VI). The superficial – deep boundary values were obtained from Nissl stained lumbar sections at each age (Torsney and Fitzgerald 2002).

2.2.2.3 Characterisation of cells and application of gabazine

Only cells with a receptive field (RF) on the plantar region of the hind paw were used. Mechanical thresholds were determined by applying calibrated nylon hairs [von Frey (vF) hairs] to the most sensitive part of the RF, with the threshold defined as the lightest hair to evoke firing of at least one action potential. The vF hairs were calibrated by applying each one to a sensitive electronic balance and measuring the force produced (in grams for simplicity). Figure 2.3 and table 2.1 show this conversion from vF hair number to grams for the set used.

Baseline recordings were made over a period of at least 10 minutes and if the activity recorded was not stable, the cell was rejected. The following parameters were measured:

(i) spontaneous activity (over a 1 minute period),

(ii) von Frey (vF) hair threshold,

(iii) receptive field area (RF area), mapped with natural mechanical stimuli:

- pointed cotton swab (light touch and brush) and forceps (noxious pinch),

(iv) spikes fired to a 3 s application of the threshold vF hair, and

(v) spikes fired to a 3 s application of the suprathreshold vF hair (3 hairs above threshold).

It was possible to measure all of these parameters in 90% of the cells recorded; a subset was measured in the remaining few cells. Threshold and suprathreshold vF hairs were chosen as the mechanical stimulus because the force exerted by vF hairs is reproducible at every application, and because vF hairs are not tissue damaging, even at the youngest age.

However, it should be noted that this type of stimulation is punctate and mechanical, and is unlikely to be noxious even at 3 hairs above threshold. Stimulation was limited to two vF hairs only (threshold and suprathreshold) due to time constraints associated with the experimental procedure.

After the baseline recordings were complete, the mineral oil was removed from the surface of the cord by laying a wick of absorbent tissue at the caudal end of the laminectomy. The wick was discarded and 5 µl of 20 µM gabazine (SR-95531; Sigma) in saline was applied topically to the exposed cord, forming a pool of liquid on the surface. This is likely to represent a maximal concentration of gabazine, as previous *in vitro* work from our laboratory and others has documented that 3 – 10 µM concentrations of gabazine abolished synaptic $GABA_AR$-mediated currents in both the immature and mature dorsal horn (Chery and De Koninck 1999). Except for a single cell at P3 (see section 2.3.2.3) gabazine was applied only once in each preparation to prevent contamination of baseline recordings with gabazine from prior applications. Hence the effect of gabazine was measured in only one cell per animal, and no rinsing was required. Spontaneous activity (measured over 1 minute), RF area, and response to threshold and suprathreshold vF hairs were measured at 5, 10, 20, 30, 40, 50 and 60 minutes after gabazine application. It was not always possible to maintain the recording of a cell for the full 60 minutes, but 75% of cells were recorded for at least 40 minutes after gabazine. The criterion used to classify a cell as showing an increase or a decrease in responsiveness after gabazine was a ≥20% change from baseline sustained over a minimum of two consecutive time points.

Response to noxious heat was tested in a separate experiment by applying steady 4 s jets of 48°C water to the centre of the RF both before (baseline) and 10 min after application of gabazine. A 30°C jet was used to control for activation of pressure and touch receptors. A minimum of two baseline measurements 10 minutes apart (at both 30°C and 48°C) were taken for each cell to ensure a stable baseline and to rule out the possibility of sensitisation with repeated application of noxious heat. The duration of the response to noxious heat varied

considerably from cell to cell, so the total number of spikes fired in response to each stimulus was counted, with the length of the window for spike counting kept constant within each cell. Heat-evoked firing rates (Hz) were also measured and were monitored in 0.5 s bins throughout the window for spike counting.

2.2.3 Analysis

Data files were recorded on an Apple Macintosh iMac running Mac OS 9.2 using Chart 4 software (AD Instruments) in conjunction with the PowerLab 4SP unit. Preliminary analyses (spike counts and peristimulus time histograms) were carried out with the Chart Spike Histogram extension, and further analyses were conducted in Microsoft Excel and GraphPad Prism. Data are presented as mean +/- standard error of the mean (SEM), unless otherwise stated. The Mann Whitney test, Wilcoxon matched pairs test, Student's t test, paired t test, two-way analysis of variance (ANOVA), and the χ^2 test were used to test for statistical significance as appropriate, and noted where applicable in section 2.3. In figures throughout this thesis, asterisks represent significance as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

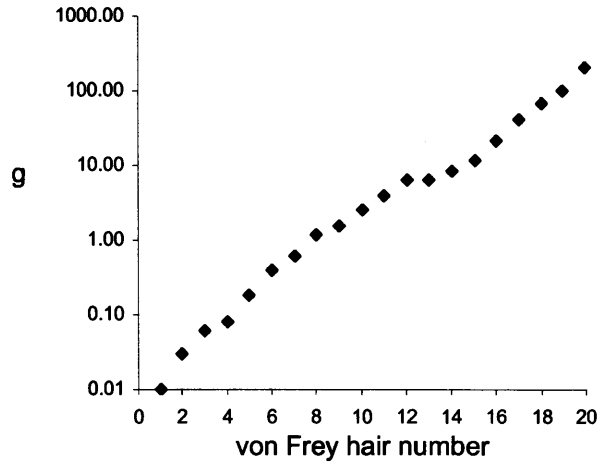


Figure 2.3 Conversion from von Frey hair number to grams
Conversion for the set of von Frey hairs used throughout this thesis.

von Frey hair number	g
1	0.01
2	0.03
3	0.06
4	0.08
5	0.18
6	0.40
7	0.60
8	1.17
9	1.55
10	2.50
11	4.00
12	6.40
13	6.40
14	8.50
15	12.00
16	21.00
17	42.00
18	70.00
19	100.00
20	200.00

Table 2.1 Conversion from von Frey hair number to grams
Conversion for the set of von Frey hairs used throughout this thesis.

2.3 Results

2.3.1 Electrophysiological properties of neonatal dorsal horn cells

Single unit recordings were made from a total of 144 cells at two ages: P3 (n = 73) and P21 (n = 71). Mean recording depths, as measured from the surface of the white matter, were 288.5 +/- 16.7 μm at P3 and 420.8 +/- 17.1 μm at P21. A slightly higher proportion of superficial cells was recorded at P3 (21/73; 28.8%) than at P21 (11/71; 15.5%; see figure 2.4).

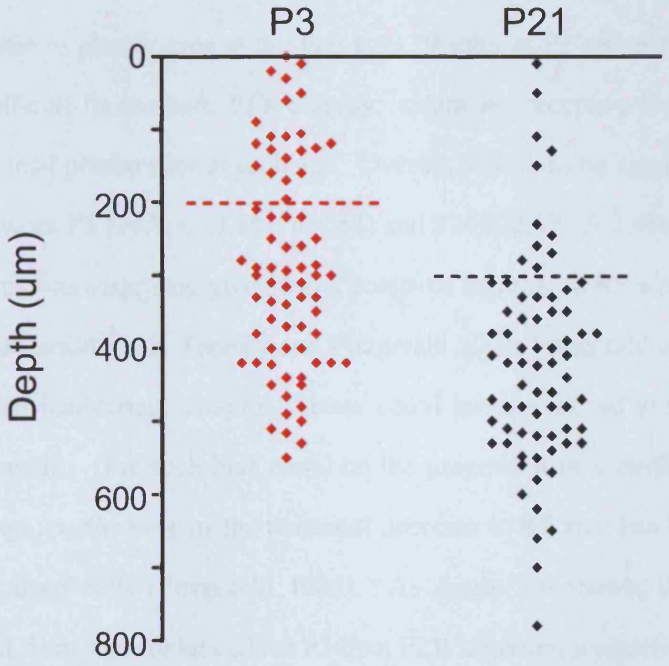


Figure 2.4 Depths of all cells recorded under urethane anaesthesia
Depths of cells at postnatal day (P) 3 (red; n = 73) and P21 (black; n = 71). Dashed lines indicate the boundary between superficial and deep cells at each age.

2.3.1.1 Mechanical thresholds

The mean mechanical thresholds as measured with von Frey hairs are shown in figure 2.5a and were 0.42 +/- 0.09 g at P3 (n = 57) and 0.60 +/- 0.10 g at P21 (n = 62). The difference in threshold between the two ages was statistically significant (Mann Whitney p = 0.026), in agreement with previous studies (Torsney and Fitzgerald 2002). There was no effect of depth on mechanical threshold at either age (see figure 2.5b).

2.3.1.2 Cutaneous receptive field size

The mean receptive field (RF) sizes were 2.35 +/- 0.3 mm^2 at P3 and 9.48 +/- 1.0 mm^2 at P21. The large difference in plantar area at the two ages (9 mm^2 at P3 compared with 42 mm^2 at P21) makes it difficult to compare RFs directly: cutaneous receptive fields were therefore normalised to the total plantar area at each age. Overall, there was no significant difference in mean RF size between P3 (25.8 +/- 3.1%; n = 53) and P21 (22.57 +/- 2.4%; n = 56), as shown in figure 2.6a. This was surprising given that a postnatal increase in RF size has been reported several times (Fitzgerald 1985; Torsney and Fitzgerald 2002; Fitzgerald and Jennings 1999). It was possible that inadvertent sampling biases could have produced a misleading result in the current experiment. One such bias could be the proportion of superficial and deep cells sampled at each age, particularly as the postnatal decrease in RF area has been reported to be most marked for deep cells (Fitzgerald 1985). As figure 2.4 shows, there was a greater tendency to record from superficial cells at P3 than P21; however, a significant difference was still not observed when the analysis was repeated with only the cells from deep dorsal horn (P3: 29.2 +/- 3.9%; n = 37; P21: 22.4 +/- 2.6%; n = 48; figure 2.6b). It should also be noted that at both ages some RFs probably extended up from the plantar surface onto the lower limb. However, stability issues with the recording preparation precluded the mapping of RFs on the lower limb, so mean RF size is likely to be an underestimate at both ages.

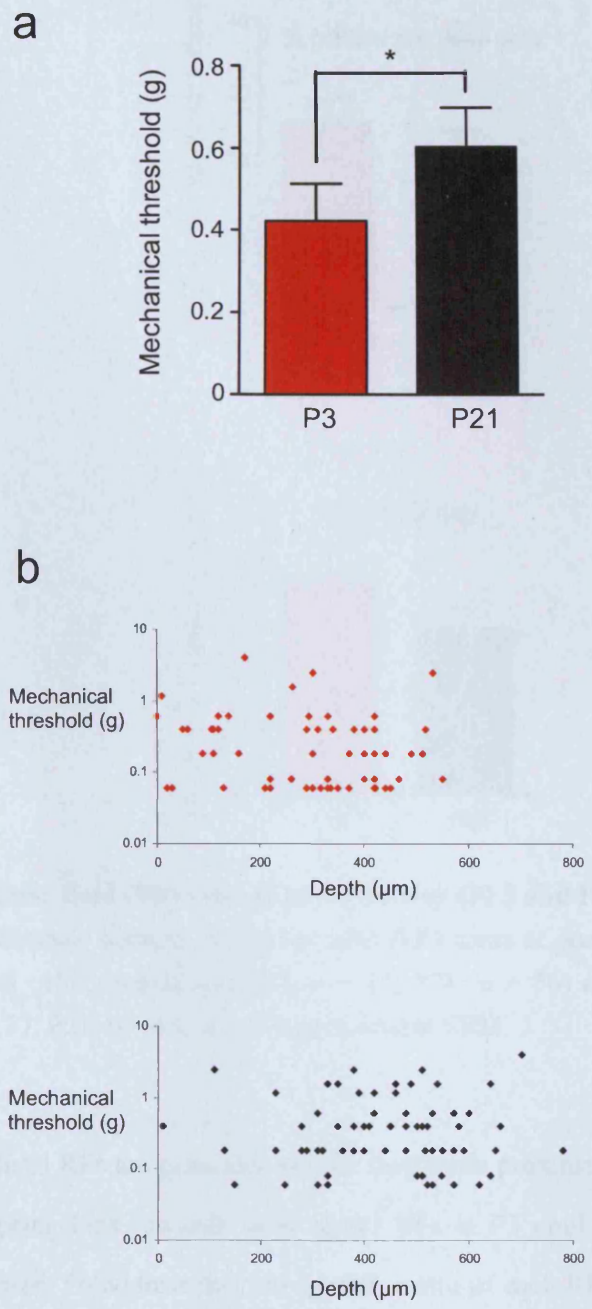


Figure 2.5 Mechanical thresholds increase with postnatal development

(a) Red and black bars show the mean von Frey hair thresholds at postnatal day (P) 3 (n = 57) and P21 (n = 62) respectively. Error bars represent SEM. (b) There was no correlation between cell depth and von Frey hair threshold at P3 (top) or P21 (bottom).

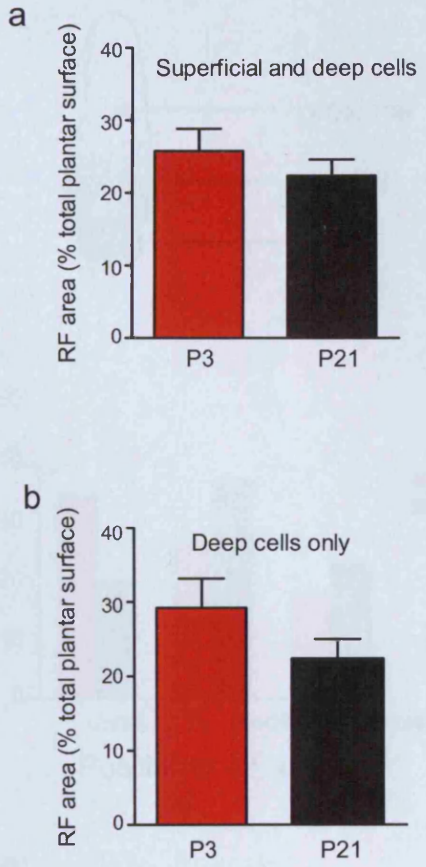


Figure 2.6 Receptive field (RF) sizes at postnatal day (P) 3 and P21
No significant difference between receptive field (RF) areas at postnatal day (P) 3 and P21 when either (a) all cells are analysed (P3: n = 53; P21: n = 56) or (b) deep cells only are analysed (P3: n = 37; P21: n = 48). Error bars represent SEM.

It is known that distal RFs are generally smaller than more proximal ones (Wilson and Snow 1988), so a sampling bias towards more distal RFs at P3 could mask a developmental difference in RF size. To address this, the spatial centre of each RF was located by eye and each RF was classified as proximal, medial or distal according to the position of the RF centre on the plantar surface (see figure 2.7a). Figure 2.7b shows that there was indeed a tendency to record from cells with more distal RFs at P3 compared with P21, which could have skewed the overall results. Figure 2.7c illustrates the clear main effect of position on RF size at both ages (two-way ANOVA: $p<0.0001$), and also shows that when position of RF is taken into

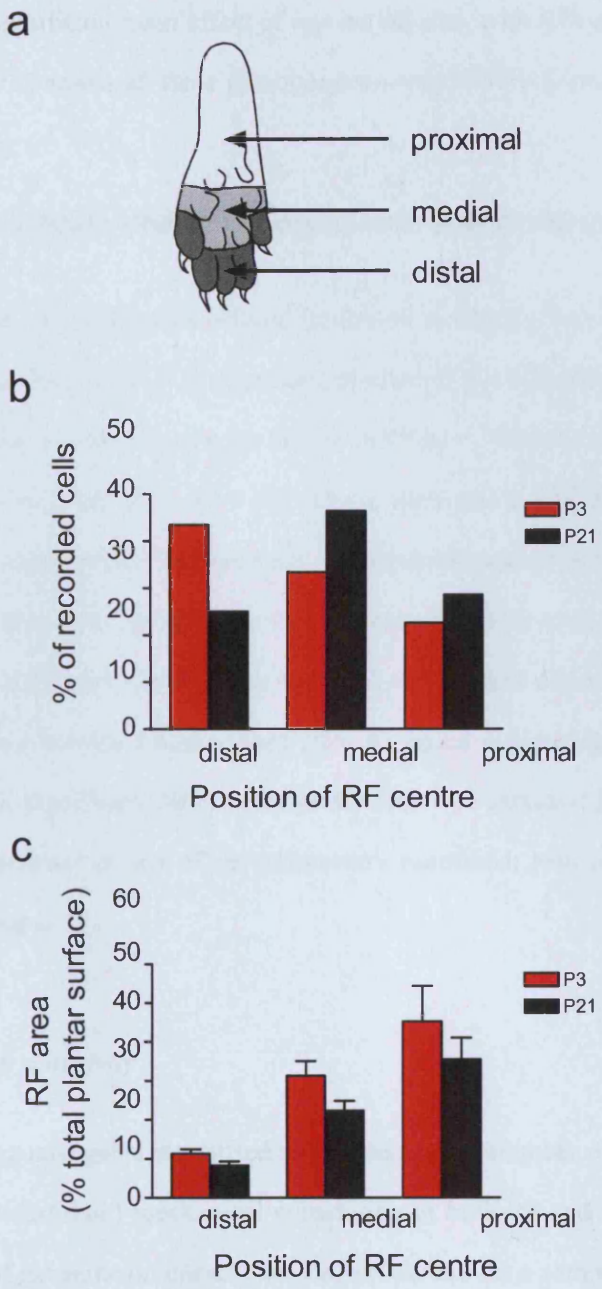


Figure 2.7 Receptive field (RF) sizes at postnatal day (P) 3 and P21 taking RF position into account

Receptive field (RF) areas are significantly larger at postnatal day (P) 3 (red) than P21 (black) when RF position is taken into account. (a) Diagram to indicate distal, medial and proximal plantar regions. (b) There was a larger proportion of cells with distal RFs at P3 than P21, and smaller proportions of cells with medial and proximal RFs. (c) At both ages, RF size increases as RF centre moves proximally ($p < 0.0001$). A two-way ANOVA shows a significant main effect of age: RFs are larger at P3 than at P21 ($p < 0.05$).

account there is a significant main effect of age on RF size, with RFs at P3 being consistently larger than RFs at P21 across all three positions (two-way ANOVA: $p<0.05$).

2.3.2 $GABA_A$Rs mediate inhibition of dorsal horn cells *in vivo* at P3 and P21

The functional state of $GABA_A$R-mediated inhibition during the postnatal period was tested by applying the specific $GABA_A$R antagonist, gabazine (5 µl of 20 µM), to the spinal cord and recording the activity of dorsal horn cells *in vivo* at P3 (n = 20 for mechanical sensitivity; plus n = 6 for noxious heat) and P21 (n = 12). These were stable single cell recordings over a period of up to 60 minutes to allow baseline and repeated post-drug testing. The proportions of superficial and deep cells sampled for mechanical sensitivity were similar for the two age groups: 8/20 cells (40%) at P3 and 4/12 cells (33%) at P21 were superficial. Single cells were isolated and baseline activity characterised prior to spinal application of gabazine. At both ages, there were no significant differences in the effect of gabazine between superficial and deep dorsal horn neurons in any of the parameters examined; results were therefore pooled from these two groups.

2.3.2.1 Mechanical sensitivity

The $GABA_A$R antagonist gabazine caused an increase in the number of spikes evoked by both threshold and suprathreshold mechanical stimulation at both P3 and P21. Figure 2.8 shows the typical effect of gabazine on dorsal horn cell properties for a sample cell at P3 and at P21. The raw spike traces below the receptive field diagrams show the response of each cell to suprathreshold vF hair stimulation [three von Frey hairs (vFh) above threshold] at baseline and then 5, 10, 30 and 60 minutes after application of the antagonist. In both the P3 and the P21 cells illustrated, there was a reversible increase in the number of spikes evoked in response to the stimulation, with a return to near baseline values by 60 minutes. Figures 2.9 and 2.10 show the receptive fields and responses to suprathreshold vF hair stimulation at baseline and at the peak of gabazine effectiveness for six typical cells at both P3 and P21.

Again, at both ages, gabazine produced a marked increase in evoked firing in most cells (see below).

The population data showed a similar pattern (see figure 2.11). The mean threshold vF hair response increased from 3.1 ± 1.0 spikes to 6.0 ± 1.4 spikes at P3 and from 12.7 ± 3.9 spikes to 19.7 ± 5.0 spikes at P21. The mean suprathreshold vF hair response increased from 9.1 ± 1.5 spikes to 15.8 ± 3.0 spikes at P3 and from 21.7 ± 3.8 spikes to 37.4 ± 8.0 spikes at P21. At both P3 and P21, there was a significant difference between baseline and peak gabazine response for both threshold and suprathreshold stimulation (Wilcoxon matched pairs tests: $p<0.01$ for all tests). No cells at any age showed a decreased response after gabazine. The proportions of responding cells were similar across age groups [threshold vF hair responders: 17/20 cells (85%) at P3 and 9/11 cells (82%) at P21; suprathreshold vF hair responders: 13/20 cells (65%) at P3 and 8/11 cells (73%) at P21; χ^2 tests: $p>0.2$ for both tests]. The mean percentage change from baseline after gabazine was not different for P3 and P21 (threshold vF hair spikes: 257 ± 41% change at P3 and 190 ± 29% change at P21; suprathreshold vF hair spikes: 183 ± 18% change at P3 and 183 ± 26% change at P21; Student's t tests: $p>0.25$ for both tests).

Background firing developed or increased after gabazine in 6/20 P3 cells but only 1/12 P21 cell, and was unaffected in the remainder.

2.3.2.2 Receptive field area

Gabazine also produced an increase in the RF area at both P3 and P21. Figure 2.8 shows the change in RF size for a sample cell at each age. The red regions indicate the baseline RF area and the yellow regions indicate the area of RF expansion at 5, 10, 30 and 60 minutes after gabazine application. Again, the effect of the drug was reversible, with the RF area returning to baseline after 60 minutes. As mentioned above, figures 2.9 and 2.10 show the RFs and responses to suprathreshold vF hair stimulation at baseline and at the peak of gabazine effectiveness for six typical cells at both P3 and P21. Again, at both ages, gabazine produced

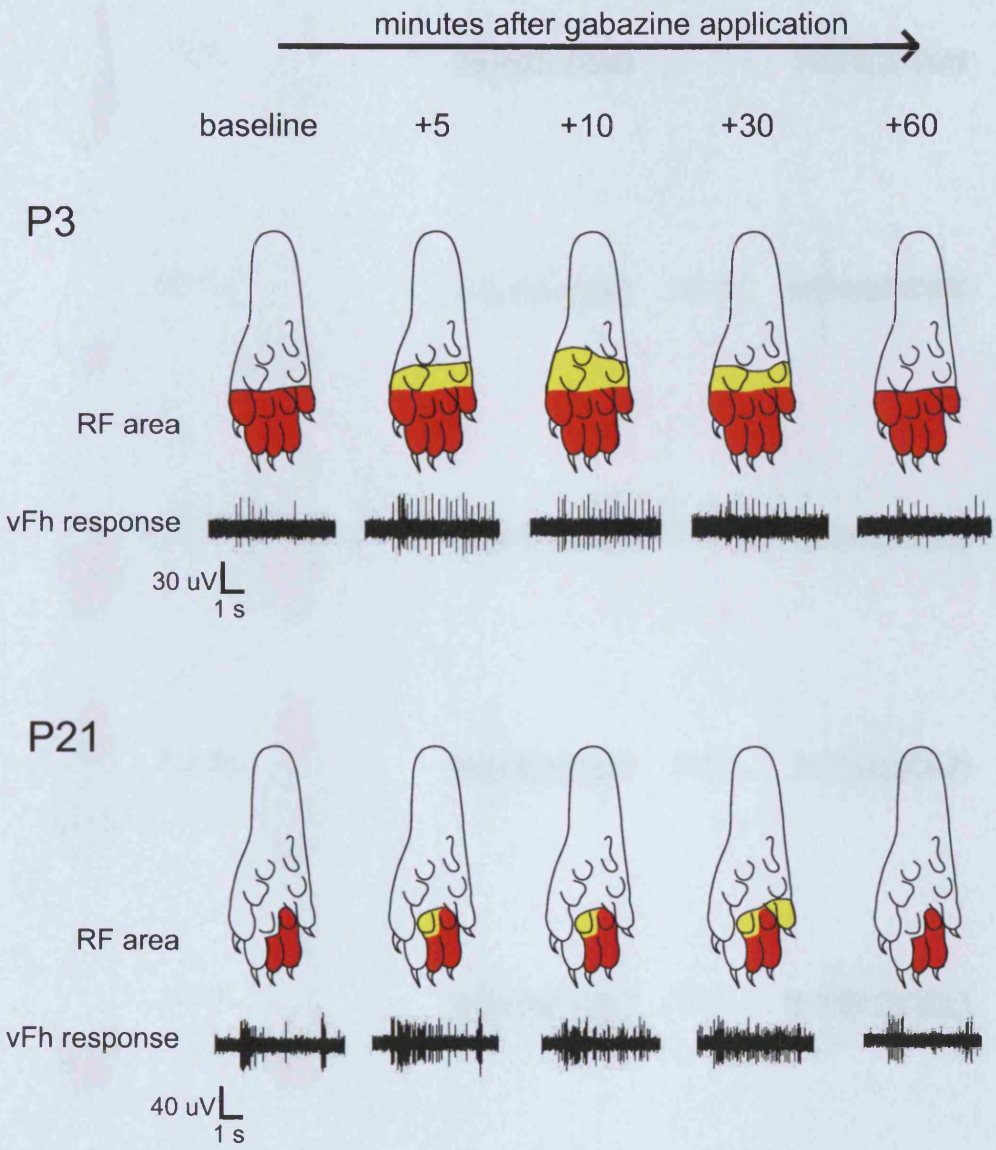


Figure 2.8 Representative cells at postnatal day (P) 3 (top) and P21 (bottom) showing the receptive field (RF) area and evoked response to suprathreshold von Frey hair (vFh) stimulation at baseline and at 5, 10, 30 and 60 minutes after gabazine. Red denotes the baseline RF area and yellow the regions of expansion.

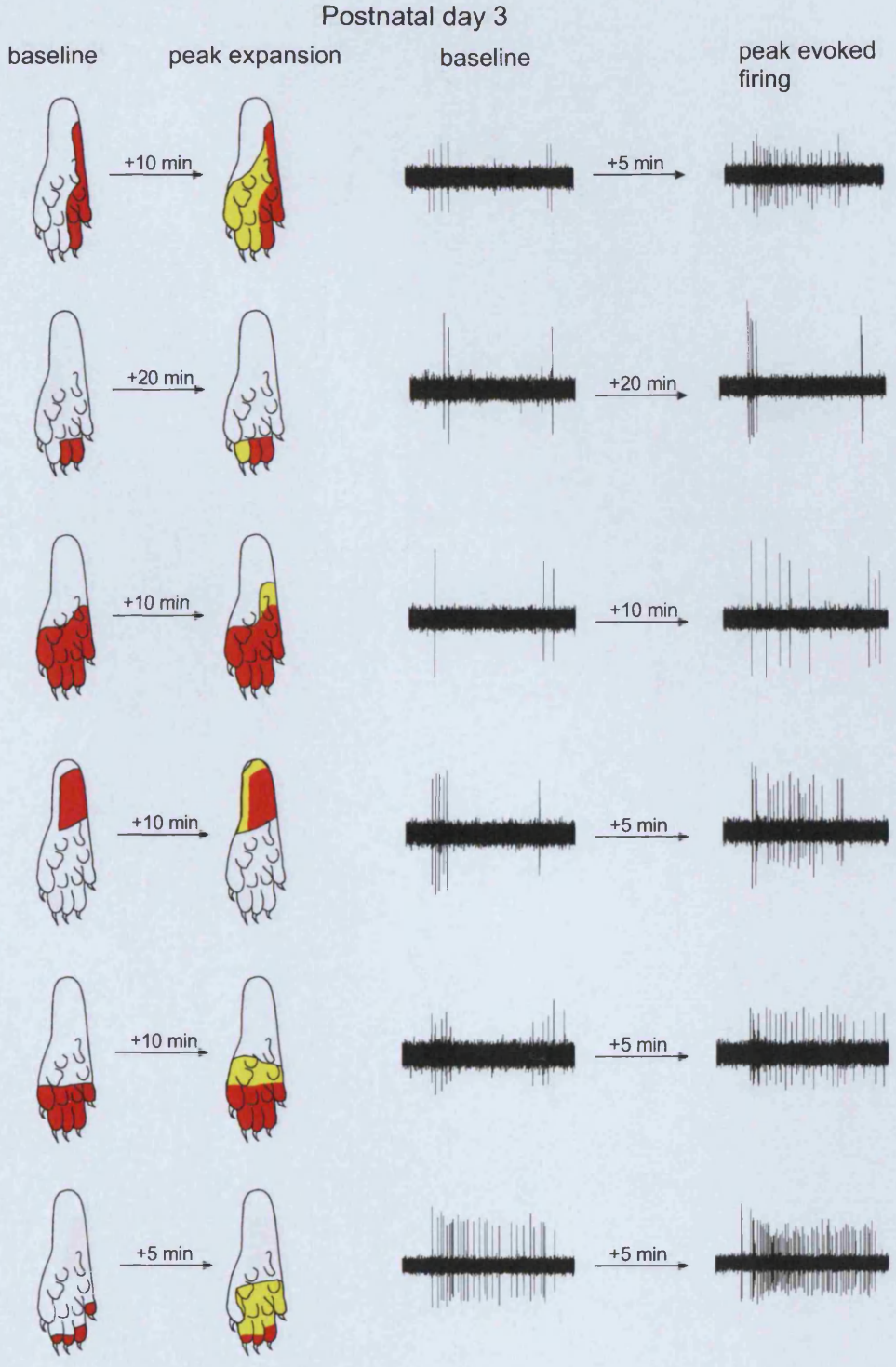


Figure 2.9 Sample receptive fields (RFs) and evoked activity after gabazine at P3
Receptive fields and spikes evoked by a suprathreshold von Frey (vF) hair for six sample cells at postnatal day 3 (P3). Baseline and peak changes after gabazine are illustrated, together with the length of time between gabazine application and the peak response for each parameter. Red denotes baseline RF area, and yellow the regions of expansion.

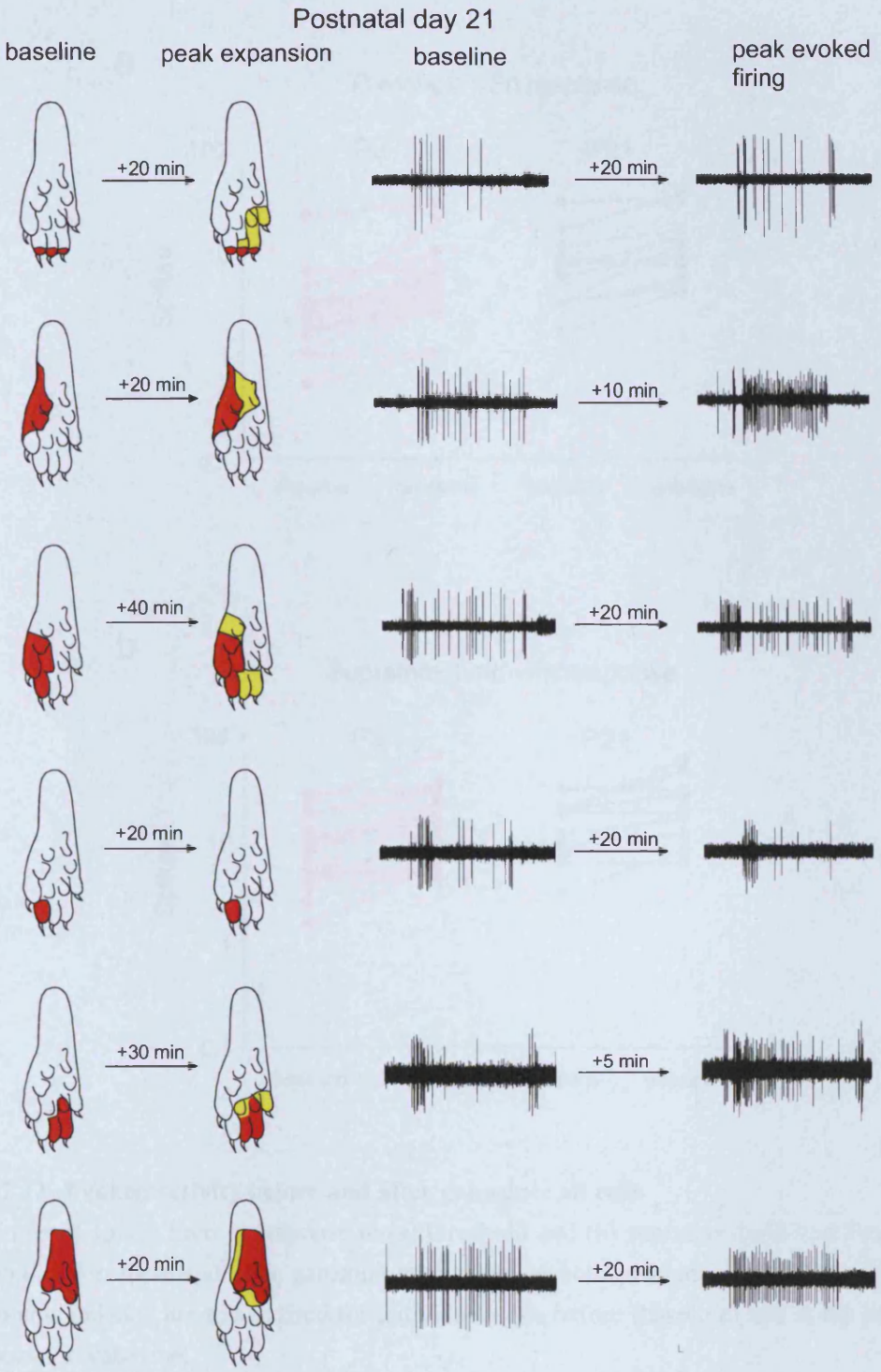


Figure 2.10 Sample receptive fields (RFs) and evoked activity after gabazine at P21
Receptive fields and spikes evoked by a suprathreshold von Frey (vF) hair for six sample cells at postnatal day 21 (P21). Baseline and peak changes after gabazine are illustrated, together with the length of time between gabazine application and the peak response for each parameter. Red denotes baseline RF area, and yellow the regions of expansion.

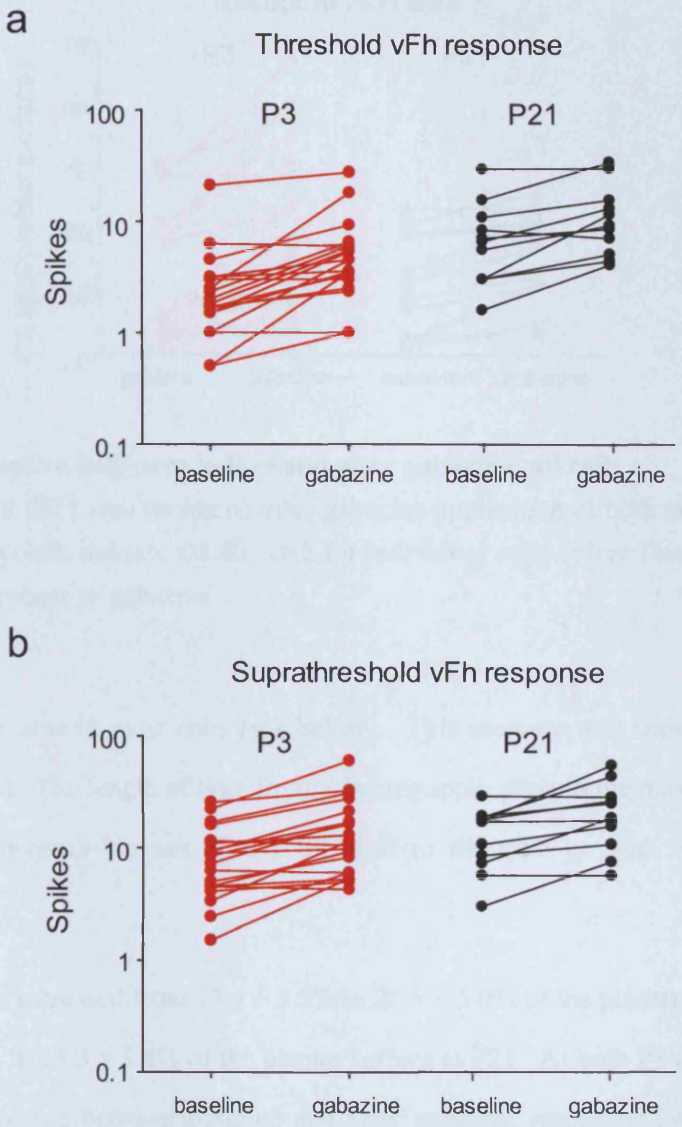


Figure 2.11 Evoked activity before and after gabazine: all cells

The number of spikes fired in response to (a) threshold and (b) suprathreshold von Frey hair (vFh) application increased after gabazine application at both postnatal day (P) 3 and P21. Paired points indicate the spikes fired for individual cells before (baseline) and at the peak of the response to gabazine.

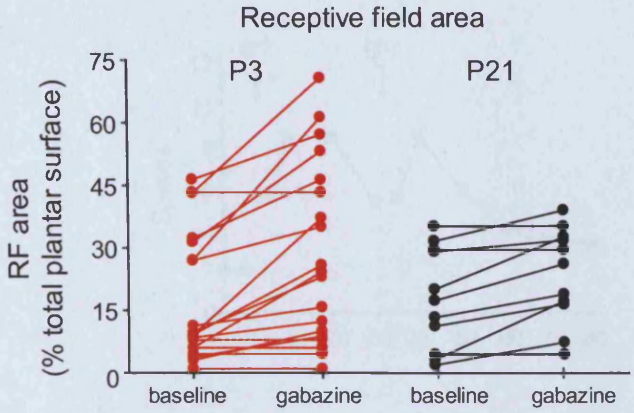


Figure 2.12 Receptive field area before and after gabazine: all cells
The receptive field (RF) area increased after gabazine application at both postnatal day (P) 3 and P21. Paired points indicate the RF area for individual cells before (baseline) and and at the peak of the response to gabazine.

an increase in RF area in most cells (see below). This increase was sometimes slight, but often quite marked. The length of time from gabazine application to the maximum increase in RF area was sometimes but not always identical to the time to peak change in evoked response.

The mean RF area increased from 17.4 ± 3.5% to 28.6 ± 5.0% of the plantar surface at P3 and from 17.8 ± 3.6% to 23.3 ± 3.4% of the plantar surface at P21. At both P3 and P21 there was a significant difference between baseline and peak gabazine responses (Wilcoxon matched pairs test: $p<0.01$ for both tests; see figure 2.12). No cells at any age showed a decreased RF area after gabazine. The proportions of responding cells were similar across age groups: 14/19 cells (74%) at P3 and 8/12 cells (67%) at P21 showed an increase in RF area (χ^2 test: $p>0.2$). The mean percentage changes from baseline in RF area or mechanical response after gabazine were not different for P3 and P21 (RF area: 191 ± 23% at P3 and 178 ± 39% at P21; Student's t test $p>0.75$).

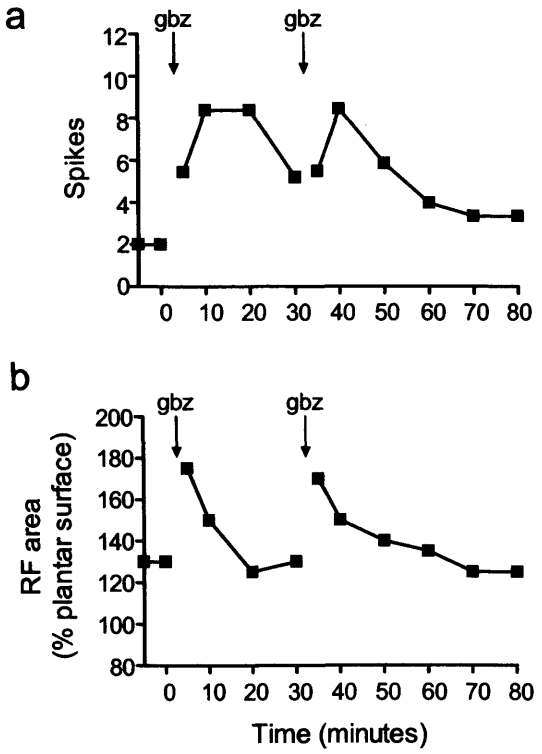


Figure 2.13 Repeatability of the effect of gabazine
Gabazine (gbz) was applied twice during the recording of one P3 cell (arrows). The spikes fired in response to von Frey (vF) hair stimulation (a) and receptive field (RF) area (b) both increased after each application.

2.3.2.3 Time-course and repeat effect

The excitatory effect of gabazine was apparent by 10 minutes post-application for both age groups, and a recovery back towards baseline for at least one of the parameters was seen in 9/14 P3 cells and 6/8 P21 cells. In one P3 cell, the drug was applied a second time, 35 min after the first application and a repeat increase in both RF area and response to vF hair stimulation was seen, illustrating the repeatability of the excitatory effect (figure 2.13).

2.3.2.4 Noxious heat

The effect of gabazine on the response to noxious heat was tested in six separate cells at P3 only. Similar to the results for mechanical stimulation (see section 2.3.2.1 above), the $GABA_AR$ antagonist produced an increase in firing evoked by noxious heat. Figure 2.14a shows sample peristimulus time histograms for the response of a cell to heat before and after gabazine application. The total number of spikes fired in response to heat increased significantly after gabazine from 47.3 ± 17.9 spikes to 81.2 ± 26.5 spikes (paired t test: $p<0.05$; see figure 2.14b). In 4/6 cells at P3, the maximum heat-evoked firing rate was increased by gabazine, from 12.5 ± 3.8 Hz to 20.0 ± 5.6 Hz. Afterdischarge duration more than doubled after gabazine in each of these four cells. The remaining two cells were unaffected. In all cases, firing to a 30°C water jet (to control for mechanical effects of the stimulation) was minimal (1.2 ± 1.2 spikes before and 1.8 ± 1.4 spikes after gabazine).

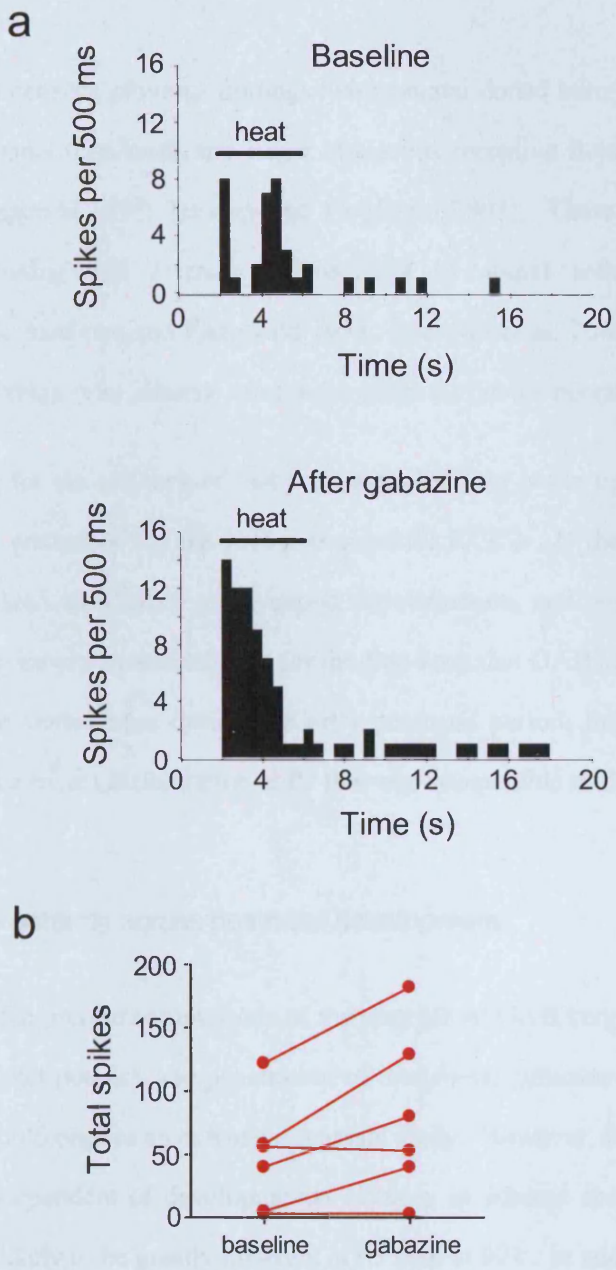


Figure 2.14 Effect of gabazine on noxious-heat-evoked firing at postnatal day 3
(a) Peristimulus time histogram showing firing of a representative heat-responsive cell before (top) and after (bottom) gabazine application. (b) The total number of spikes fired in response to the 4 s heat stimulus increased after gabazine in 4 of 6 heat-responsive cells. Measurements were taken at baseline and at 10 minutes after application of gabazine.

2.4 Discussion

The present results confirm previous findings that neonatal dorsal horn cells recorded *in vitro* have lower mechanical thresholds and larger cutaneous receptive fields than those found in older animals (Fitzgerald 1985; Torsney and Fitzgerald 2002). These results, together with reduced spatial tuning and increased excitability of spinal reflexes (Holmberg and Schouenborg 1996; Andrews and Fitzgerald 1994; Andrews et al. 2002), are consistent with the hypothesis that there is an absence of or weak inhibition in the neonatal dorsal horn.

One critical factor for the efficacy of fast synaptic inhibition is the transmembrane chloride gradient, which is controlled by the KCl cotransporter KCC2. If the chloride extrusion is immature, then GABA may cause postsynaptic depolarisation, and possibly even excitation. The present results, however, demonstrate for the first time that GABAergic inhibition *in vivo* is functional in the dorsal horn during the early postnatal period: the $GABA_AR$ antagonist gabazine produced a robust disinhibition at P3 that was comparable to that seen at P21.

2.4.1 Gabazine potency across postnatal development

It is difficult to make precise comparisons of the strength of GABAergic inhibition at P3 and P21 because the exact potency and penetration of intrathecal gabazine at the two ages is not known, and this would require an extensive separate study. However, the potency of gabazine is known to be independent of developmental changes in subunit composition (Ebert et al. 1997) and so is unlikely to be greatly different at P3 than at P21. In addition, the dose used (5 µl of 20 µM) was greater than the concentration shown to abolish synaptic $GABA_AR$-mediated currents in both neonatal and adult dorsal horn *in vitro* (3 – 10 µM; (Chery and De Koninck 1999), so can reasonably be assumed to be a supramaximal dose at both ages. Therefore, the fact that the gabazine-induced disinhibition appeared at least as strong at P3 as at P21 suggests that GABAergic inhibition is relatively mature in the dorsal horn by the third postnatal day.

2.4.2 Comparisons with the rest of the CNS

It appears from the results in this chapter that GABAergic inhibition matures earlier in the dorsal horn than has been reported for many other areas of the CNS. For example, numerous studies have shown that GABA does not become inhibitory in the hipppocampus until the second postnatal week, and a similar time course has been reported in auditory brainstem, cerebellum, and cortex (Rivera et al. 1999; Balakrishnan et al. 2003; Zhu et al. 2005; Ehrlich et al. 1999). However, relatively early maturation of functional inhibition has previously been reported for some CNS regions, including respiratory centres in the brainstem (Ikeda et al. 2004). Within the spinal cord itself, GABA reduces the excitability of motoneurons in the ventral horn by embryonic day 18.5 (Hubner et al. 2001). It appears therefore that in some CNS regions crucial for immediate postnatal survival, such as those mediating respiration and movement, early maturation of inhibition is necessary to allow these regions to function correctly. CNS areas such as the hippocampus, however, may benefit from the additional neurogenesis which is thought to be driven by GABAergic excitation during the first postnatal week. It is also important to note that, unlike the hippocampus where the excitatory glutamatergic synapses only become functional during the second postnatal week, a glutamatergic drive is present in the spinal cord at early postnatal ages (Baccei et al. 2003) and a complete absence of GABAergic inhibition could result in seizure-like activity.

2.4.3 Shunting inhibition may be sufficient at P3

The strong GABAergic inhibition at P3 was surprising given that the levels of KCC2 protein in the dorsal horn are very low at this age (E. Harrop and M. Fitzgerald, unpublished observations; see figure 2.2) and that $GABA_AR$-mediated depolarisations and calcium influx are observed in a proportion of superficial dorsal horn cells in the early neonatal spinal cord (Baccei and Fitzgerald 2004; Cordero-Erausquin et al. 2005). From these previous studies, the simplest prediction would be that the inhibition at P3 should be substantially weaker

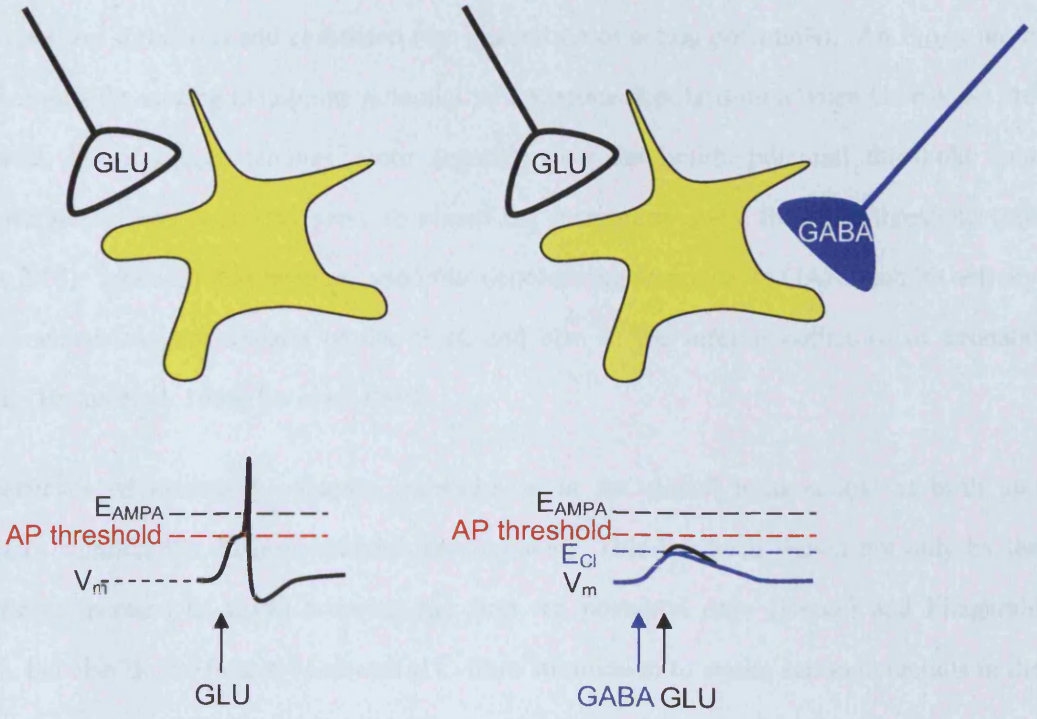


Figure 2.15 Diagram of shunting inhibition
Activation of AMPA receptors by glutamate drives the membrane potential towards the threshold for firing action potentials (left). If $GABA_A$Rs are activated, this can also cause a depolarisation of the membrane in immature neurons. Despite this, $GABA_A$R activation may still inhibit the effects of glutamate as the increased membrane conductance can serve to clamp the membrane potential below the action potential threshold (right).

than that seen at P21, when GABA always evokes membrane hyperpolarisation. However, this was not observed in the *in vivo* experiments described in this chapter.

One explanation for this apparent discrepancy is that inhibition through both $GABA_A$Rs and GlyRs is not mediated solely by the hyperpolarisation carried by influx of chloride ions. The opening of channels in the membrane also significantly increases the membrane conductance, and this decreases overall neuronal excitability by shunting subsequent excitatory inputs (Bormann et al. 1987). This shunting component of the inhibition is independent of the transmembrane chloride gradient and so would be expected to be present at all ages. It is also

important to draw a distinction between depolarisation (a movement of the membrane voltage in the positive direction) and excitation (the generation of action potentials). An E_{GABA} more positive than the resting membrane potential will produce depolarisation when $GABA_A$Rs are activated, but if E_{GABA} remains more negative than the action potential threshold then GABAergic activity may still serve to clamp the membrane away from the threshold (see figure 2.15). Indeed, it has been reported that depolarising responses to GABA inhibit activity in the nucleus magnocellularis of the chick and also in the inferior colliculus of neonatal gerbils (Hyson et al. 1995; Lo et al. 1998).

The efficacy of excitatory synaptic transmission in the dorsal horn is low at birth and increases significantly during postnatal development. This has been shown not only by the significant increase in sEPSCs during the first ten postnatal days (Baccei and Fitzgerald 2004), but also by the failure of electrical C-fibre stimulation to evoke action potentials in the dorsal horn until ~P10 (Jennings and Fitzgerald 1998). It is possible that the increase in conductance during activation of $GABA_A$Rs is sufficient to effectively shunt these lower levels of excitation during the first few postnatal days, despite a deficit in strong hyperpolarisation.

The protein expression levels of KCC2 can be a useful indicator of inhibitory function in some areas of the CNS: in the hippocampus, for example, the time course of maturation of inhibition closely matches the time course of KCC2 upregulation (Stein et al. 2004). However, the present results show that levels of KCC2 should not be used as a sole predictor of inhibitory function. This is supported by a recent finding that KCC2 must form oligomers in order to be a functional 'transport active' cotransporter (Blaesse et al. 2006). Hence absolute membrane levels of KCC2 do not necessarily indicate levels of active protein, and electrophysiological experiments are necessary to accurately determine the state of inhibitory processing.

2.4.4 Chloride extrusion and intense stimulation

It has been reported that although the shift in resting E_{GABA} is complete by the end of the first postnatal week, chloride extrusion does not reach full capacity in lamina I neurons until the end of the third postnatal week (Cordero-Erausquin et al. 2005). This is consistent with the developmental upregulation of KCC2, which reaches adult levels by P21. The effect of this immature chloride extrusion was observed only during prolonged stimulation with either exogenous GABA or trains of stimulation at 20 - 100 Hz, during which a gradual shift in the polarity of the response was seen from hyperpolarising at the start to depolarising towards the end. However, the present results suggest that the low chloride extrusion at P3 is sufficient to maintain adequate inhibition in the dorsal horn, as the disinhibition observed with the $GABA_AR$ antagonist was similar at P3 as at P21.

The surgical anaesthesia necessary for the experiments described in this chapter results in low rates of firing, even in response to suprathreshold mechanical stimulation, so it is possible that a different result with gabazine would have been seen with more intense stimulation. The main experiment was therefore repeated using prolonged noxious heating (48°C for 4 s) of the receptive field to produce greater drive to the dorsal horn (see section 2.3.2.4). It might be predicted that increased GABAergic activity concurrent with the initial burst of firing during the heat stimulus might result in a decrease in intracellular $[Cl^-]$ at P3, and a prolonged subsequent afterdischarge. Such afterdischarges are in fact commonly seen after stimulation in neonatal animals (Fitzgerald 1985; Jennings and Fitzgerald 1998). While gabazine was present in the dorsal horn, $GABA_ARs$ were blocked so there could be no activity-induced change in intracellular $[Cl^-]$. This makes interpretation of the results with respect to the work of Cordero-Erausquin et al. (2005) non trivial; however, there was no reduction in the afterdischarge in any of the cells with gabazine, and the afterdischarge actually doubled in 4/6 cells, suggesting that gabazine again caused a robust disinhibition even to this stronger stimulus.

It is possible that lamina I cells may have a different chloride homeostasis than the mainly deeper cells recorded in the present study. In the hippocampus there is a well-described shift in GABAergic action on principal cells from excitatory to inhibitory, but a different effect of GABA is seen on a subgroup of CA3 hippocampal interneurons where inhibition remains shunting throughout development (Banke and McBain 2006), illustrating that populations of cells may regulate chloride homeostasis differentially. This would appear to be unlikely in the immature dorsal horn as no differences were observed between superficial and deep cells in this experiment, but the possibility cannot be excluded. A more likely explanation is that the frequency of spontaneous IPSCs is significantly lower and primary afferent evoked IPSCs are weaker in the early neonatal dorsal horn, so the overall chloride load imposed on the postsynaptic cell during normal sensory stimulation may be lower than that seen in the adult, despite the slower decay of GABA-mediated IPSCs at this age (Keller et al. 2004). The reduced chloride extrusion at P3 may therefore still be sufficient to allow for adequate neuronal inhibition under normal circumstances at this age. With regard to this, it should also be noted that although the present experiments were conducted *in vivo* in order specifically to examine the inhibition of activity evoked by natural stimulation in an intact animal, the presence of deep anaesthesia will have reduced the overall level of activity in the dorsal horn compared with that in an unanaesthetised animal. Hence it is possible that run-down of the intracellular [Cl^-] due to low extrusion capacity may be more likely to occur in an awake behaving animal or in a slice preparation.

Pathological conditions can result in very intense or prolonged stimulation, and it is possible that in such situations the chloride extrusion mechanisms at P3 may be unable to maintain adequate intracellular [Cl^-] and inhibition may be reduced or even reversed, as occurs *in vitro* during high frequency stimulation (Cordero-Erausquin et al. 2005). Indeed, sustained firing can result in down-regulation of KCC2 protein and subsequent disruption of chloride homeostasis, as has been demonstrated in the mature hippocampus (Rivera et al. 2004). In the dorsal horn itself, damage to peripheral nerves in the adult can also result in down-regulation of KCC2 in postsynaptic cells and the resultant shift in E_{Cl} has been proposed as an underlying

mechanism for the maintenance of neuropathic pain (Coull et al. 2003). It remains to be seen whether nerve damage also affects the chloride homeostasis in neonates.

2.4.5 Gain control

Under normal, non-pathological conditions GABAergic activity may contribute to signal processing in the dorsal horn by sharpening spatial cutaneous receptive fields and gating input from specific modalities. However, it is also likely that GABAergic inhibition acts more generally as a gain control mechanism in the dorsal horn. Gain control is essential in sensory systems because the range of neuronal firing rates is more limited than the dynamic range of the sensory input (Ohzawa et al. 1982; Ingham and McAlpine 2005). Gain control mechanisms scale the sensitivity of a system to a level appropriate for the input so that useful information can be extracted.

Since GABAergic activity was found to be functionally inhibitory in the dorsal horn by P3, it is probable that such gain control mechanisms are already in place in this system by the third postnatal day. However, this was not tested explicitly and, moreover, the type of gain control (multiplicative or additive) may alter during postnatal development, particularly if shunting inhibition is of greater relevance in the neonatal cord (Mitchell and Silver 2003). The use of only two von Frey hairs in the current study limited the analysis in this respect. Testing with a larger range of stimuli (for example, five different von Frey hairs) would enable an input – output function to be plotted at each age before and after the application of a $GABA_AR$ antagonist. It would then be possible to assess whether the antagonist shifted the slope and/or intercept of the function in the same way at each age and hence whether gain control processes in the dorsal horn alter with postnatal development.

2.4.6 Conclusions

The present results confirm that neonatal dorsal horn cells have lower mechanical thresholds and larger cutaneous receptive fields than those in the adult; however, it is unlikely that this is due to insufficient GABAergic activity in the dorsal horn. The $GABA_AR$ antagonist gabazine revealed a robust tonic inhibition is already functional in the rat dorsal horn by the third postnatal day. The increased excitability of tactile and nociceptive circuits at young ages may instead result from developmental differences in other types of local inhibitory synapses, such as the opioidergic and glycinergic systems, from immaturity in descending inhibitory systems, or from more subtle differences in the organisation of dorsal horn circuitry at the network level.

Chapter 3

Descending modulation of the immature dorsal horn

3.1 Introduction

Descending modulation plays a key role in setting the excitability of dorsal horn circuits, influencing both the perception of pain and nocifensive behaviour. In the adult, this supraspinal modulation can be both inhibitory and facilitatory, and is mediated by a plethora of interconnected anatomical pathways and transmitter / receptor systems (see section 1.4). The brainstem, and the rostral ventromedial medulla (RVM) in particular, is a major source of descending pathways, which project to the dorsal horn largely in the dorsolateral funiculus (DLF) and ventrolateral funiculus (VLF) (Basbaum and Fields 1979). Although descending facilitation is increasingly recognised as an important feature of supraspinal controls, particularly under conditions of tissue damage or nerve injury (Suzuki et al 2004), in the uninjured, anaesthetised cat and rat, the DLF is known to mediate a strong tonic inhibitory influence on dorsal horn cells and spinal reflexes (Sherrington and Sowton 1915; Wall 1967; Laird and Cervero 1990). Moreover, electrical stimulation of the DLF in adult rats inhibits C-fibre evoked firing of dorsal horn cells (Fitzgerald and Koltzenburg 1986).

3.1.1 Functional and anatomical maturation of descending modulation

There is compelling evidence that descending pathways mature in the rat over the first three postnatal weeks. An early indication of this came from the report that rats spinalised at thoracic levels when younger than postnatal day 15 (P15) were relatively unaffected by the operation compared with older animals (Weber and Stelzner 1977). Furthermore, stimulation of the midbrain in adult rats produces profound antinociception but this does not develop until approximately P21 (van Praag and Frenk 1991). Formalin injected into the hindpaw results in a biphasic behavioural response in adult rats, thought to be due in part to descending inhibition (Henry et al. 1999); however the classic biphasic response was not observed in pups younger than P15 (Guy and Abbott 1992). Diffuse noxious inhibitory controls (DNIC) are a supraspinal form of control in which the response to a stimulus is reduced if it is accompanied by a concurrent heterotopic noxious stimulus. DNIC, as measured by a reduction in c-Fos

immunoreactivity (an immediate-early gene marker of activity) to the original stimulus, was also found to be ineffective before P21 (Boucher et al 1998). Direct electrical stimulation of the DLF in rats aged from P8 to adult showed a gradual postnatal increase in the efficacy of this stimulation to inhibit C-fibre evoked activity in individual dorsal horn neurons: no cells were inhibited at P8, ~50% of cells were inhibited at P12, and ~90% of cells were inhibited from P22 onwards (Fitzgerald and Koltzenburg 1986). In addition, the reflex receptive fields remain disorganised in adult rats which were spinalised as neonates, indicating that postnatal maturation of descending controls is necessary for the tuning of nociceptive reflexes (Levinsson et al. 1999).

There have been several studies on the development of descending supraspinal pathways. Projections are known to grow down from the RVM during early foetal life and are anatomically present in the neonatal spinal cord (Gilbert and Stelzer 1979; Leong et al. 1984, Bregman 1987; Rajaofetra et al 1989; Tanaka et al. 1992, 2006). Likewise, the DLF contains an adult-like distribution of fibres descending from the RVM and other brainstem nuclei as early as P6 (Fitzgerald and Koltzenburg 1986). However, recent work suggests that the projections to the ventral horn arrive first and that projections to the dorsal horn may not develop until the postnatal period (Tanaka et al. 2006). A retrograde tracing study found that adult-like proportions of serotonergic neurons project from the nucleus raphe pallidus of the medulla to the lumbar dorsal horn at P3, but that few serotonergic neurons project from the nucleus raphe magnus (NRM) at this age (Tanaka et al. 2006). The NRM is an important source of supraspinal 5-HT modulation of the dorsal horn in adult rats, whereas the raphe pallidus projects to the ventral horn (Skagerberg and Bjorklund 1985), so these results support the notion of a ventro-dorsal developmental gradient of descending spinal pathways, at least for 5-HT-positive fibres. It is also possible that the lack of descending inhibition in neonates is not due to delayed axonal growth but is related to postnatal proliferation of local terminals of descending axons within the dorsal horn, which has been reported to occur between P3 and P21 for serotonergic (5-HT) terminals (Bregman 1987; Tanaka et al. 1992). In addition to potentially low levels of 5-HT-positive terminals in the neonatal cord, serotonergic

modulation may also be limited by delayed development of 5-HT receptors. In support of this, it has been demonstrated that a 5-HT receptor agonist administered intrathecally does not become antinociceptive until P10 (Giordano 1997). However, the developmental profiles of the multiplicity of 5-HT receptor subtypes are not yet known. In contrast, agonists at the α_2-AR are analgesic in the neonate (Hughes and Barr 1988; Walker et al. 2005), indicating that postnatal receptor maturation may be selective for 5-HT receptors. However, it should be noted that although noradrenalin (NA) receptor expression may be well-developed, levels of NA are low in the spinal cord during the early postnatal period: it has been reported that NA terminals proliferate after P3 (Tanaka et al. 1992, 1996; Aramant et al. 1986).

While descending inhibition requires some postnatal weeks to mature, maturation of descending facilitation in the neonate has not been investingated. Recent work suggested that there may in fact be a postnatal switch in descending controls from tonic facilitation in the neonate to tonic inhibition in the adult, rather than simply a postnatal upregulation in descending controls per se. Blockade of $GABA_A$Rs in the spinal cord, which is disinhibitory or excitatory in older animals, has the reverse effect, i.e. is inhibitory at P3. This developmental difference was abolished by spinalisation suggesting that descending controls are strongly regulated over the postnatal period (Hathway et al. 2006).

Electrophysiological studies on functional postnatal development of descending pathways upon dorsal horn activity have so far examined only the maturation or degree of inhibition and have not assessed facilitation; it is possible that descending excitation may have a prominent effect on processing in the neonatal dorsal horn.

3.1.2 Aims of the chapter

The aims of the experiments in this chapter were two-fold:

1) To study the postnatal development of dorsal horn cell properties *in vivo* in a decerebrate spinalised preparation using single unit extracellular electrophysiological recordings. This provided information about the baseline properties of dorsal horn receptive fields at different ages in the absence of any descending influences from the brain.

2) To investigate the influence of activating descending projections to dorsal horn circuits in the postnatal rat. The dorsolateral funiculus (DLF) was electrically stimulated at a range of amplitudes and the effects on dorsal horn cell evoked and spontaneous activity were recorded at postnatal day 3 and in the adult. This provided information about the relative strength of inhibitory and/or excitatory descending control over dorsal horn cell activity at different postnatal ages.

3.2 Materials and methods

3.2.1 Decerebrated, spinalised preparation

P3 and adult (~P42; ~180 g) Sprague Dawley rats were used in these experiments. P3 rats were anaesthetised on ice and adult rats were anaesthised with a short acting injectable anaesthetic: 0.6 ml/kg i.p. Hypnorm plus 2.5 mg/kg i.p. diazepam. This produces ~45 minutes of surgical anaesthesia (Wolfensohn and Lloyd 1998). After induction of anaesthesia, animals were tracheotomised and then mounted in a stereotaxic frame above a heated blanket. The electrocardiogram (ECG) was monitored via electrodes inserted into the fore paws. A suction decerebration was performed at the mid-collicular level, with all tissue rostral to this point removed and the skull cavity filled with Gelfoam and cotton wool to prevent haemorrhage. Anaesthesia was slowly withdrawn and the decerebration confirmed by the presence of reflexes to noxious stimulation in the absence of changes in the ECG. Following verification of the decerebration, the animals were artificially ventilated: the tracheal cannula was connected to a T-piece which supplied medical oxygen, and intermittent positive pressure ventilation at 80 strokes per minute was provided by a small animal ventilator (model 687, Harvard apparatus). Neuromuscular blockade was then induced with pancuronium bromide (2.0 mg/kg i.p.) to aid stability and produce smooth artificial ventilation. The ECG was monitored throughout the experiment.

The spinal cord was exposed at the thoracic level and sectioned to remove all descending influences and completely isolate the lumbar spinal cord. Stimulating electrodes (glass-coated tungsten, 10 um tip) were positioned bilaterally in the dorsolateral funiculus caudal to the spinalisation. The dorsal columns were crushed with a pair of forceps caudal to the electrodes to prevent antidromic stimulation.

A second laminectomy was performed over L4 – L5 to allow extracellular recordings from lumbar dorsal horn neurons, which were carried out as described in section 2.2.2.2. The recording depth from the surface was noted and cells classified as superficial (presumed

laminae I and II; depth ≤ 200 μm from the dorsal surface at P3, ≤ 350 μm in the adult) or deep (presumed laminae III, IV, V, VI). The superficial – deep boundary values were obtained from Nissl stained lumbar sections at each age (Torsney and Fitzgerald, 2002).

3.2.2 Measurement of cell properties

Cells were classified as low threshold (LT), noxious specific (NS), or wide dynamic range (WDR). Cutaneous receptive fields were mapped in 90% of recorded cells, and mechanical von Frey hair thresholds were determined in 60% of recorded cells.

3.2.3 Stimulation of descending pathways in the dorsolateral funiculus

Stimulating electrodes positioned bilaterally in the dorsolateral funiculus (DLF) were connected to a stimulus isolator unit (NL800), which in turn was controlled by a set of Neurolog modules (NL303, NL402, and NL510). Stimulating trains consisted of 150 μs-wide pulses delivered at 50 Hz for an overall duration of 5 s. A range of stimulus amplitudes was used: 20, 50, 100, 200 and 400 μA. Immediately prior to DLF stimulation, a suprathreshold vFh (3 hairs above threshold) was applied for 3 s to the centre of the receptive field and the number of spikes evoked was measured. The same vFh was then applied during the DLF stimulation, and the number of spikes evoked noted again. A stimulus artifact was usually observed during recordings with DLF stimulation; however, evoked spikes could be clearly distinguished from the artifact during analysis. For cells with spontaneous activity, the baseline firing rate (Hz) was established over the 5 s prior to DLF stimulation and compared with the firing rate during the 5 s of the stimulus train.

3.3 Results

3.3.1 Electrophysiological properties of neonatal dorsal horn cells in a decerebrate, spinalised preparation

Single unit recordings were made from a total of 192 cells at two ages: P3 (n = 115) and adult (n = 77). Mean recording depths, as measured from the surface of the white matter, were 299.1 +/- 13.4 μm at P3 and 567.4 +/- 21.4 μm in the adult. A higher proportion of superficial cells was recorded at P3 (28/115; 24.3%) than in the adult (10/77; 13.0%; see figure 3.1). The majority of cells recorded were wide dynamic range cells (WDR; P3 58.6%; adult 77.9%) with fewer nociceptive specific (NS; P3 14.4%; adult 10.3%) and low threshold cells (LT; P3 27.0%; adult 11.8%). The proportion of cells in each category changed with postnatal age (X^2 test; $p < 0.05$; see table 3.1). The lower proportion of WDR cells at P3 compared with the adult may reflect the relative lack of convergence in the dorsal horn at this young age (Fitzgerald 1985).

	WDR	NS	LT	not defined	total
P3	65	16	30	4	115
adult	53	7	8	9	68

Table 3.1 Numbers of cells in each class at postnatal day (P) 3 and in the adult
WDR - wide dynamic range (brush and pinch); NS - noxious specific (pinch only); LT - low threshold (brush only).

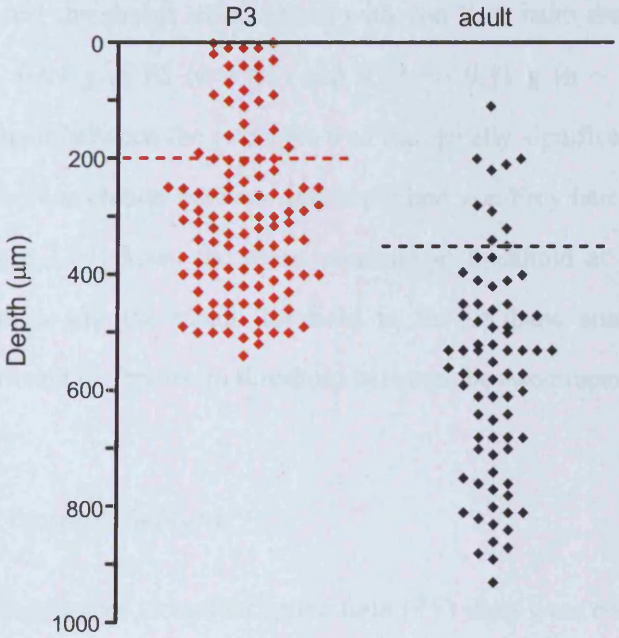


Figure 3.1 Depths of all cells recorded in the decerebrated, spinalised preparation
Depths of cells at postnatal day (P) 3 (red; n = 115) and in the adult (black; n = 77). Dashed lines indicate the boundary between superficial and deep cells at each age.

3.3.1.1 Mechanical thresholds

The mean mechanical thresholds as measured with von Frey hairs are shown in figure 3.2a, and were 0.30 +/- 0.04 g at P3 (n = 87) and 0.52 +/- 0.11 g (n = 29) in the adult. The difference in threshold between the two ages was statistically significant (Mann Whitney p = 0.02). There was no correlation between cell depth and von Frey hair threshold at either age (figure 3.2b). Figure 3.2c shows the mean mechanical threshold at P3 in the decerebrate, spinalised preparation and the mean threshold in the urethane anaesthetised preparation. There was no significant difference in threshold between the two preparations.

3.3.1.2 Cutaneous receptive field size

For comparison between ages, actual receptive field (RF) sizes were converted to a percentage of the total plantar area. In this decerebrate, spinalised preparation, the mean RF size was significantly smaller at P3 than in the adult (P3: 40.2 +/- 2.8%, n = 109; adult: 59.6 +/- 4.0%, n = 63; p = 0.0001; figure 3.3a). As in chapter 3, the RFs were classified according to whether the centre of the RF was located in a proximal, medial or distal location on the plantar surface (see figure 2.6a). The proportions of cells recorded in the three categories were not identical at the two ages (figure 3.3b), but the RF area was still significantly smaller at P3 than in the adult even when this was taken into account (2-way ANOVA, main effect of age p = 0.003; figure 3.3c). Bonferroni post-tests revealed that the difference in RF size with age was highly significant ($p < 0.001$) for the most proximal RFs. This is the opposite of the age-related results under urethane anaesthesia, in which P3 RFs were larger than P21 RFs (see section 2.3.1.2). There was also a significant main effect of position of RF centre on RF size at both ages ($p < 0.0001$), with RF size increasing as the plantar location moves proximally.

A direct comparison of RF sizes at P3 under urethane anaesthesia and in the decerebrated, spinalised preparation revealed that RFs were significantly larger in the decerebrated preparation than in the anaesthetised preparation (urethane: 25.8 +/- 3.1%, n = 53; decerebrate, spinalised 40.2 +/- 2.8%; figure 3.4a). This may be due to the larger proportion of distal RFs

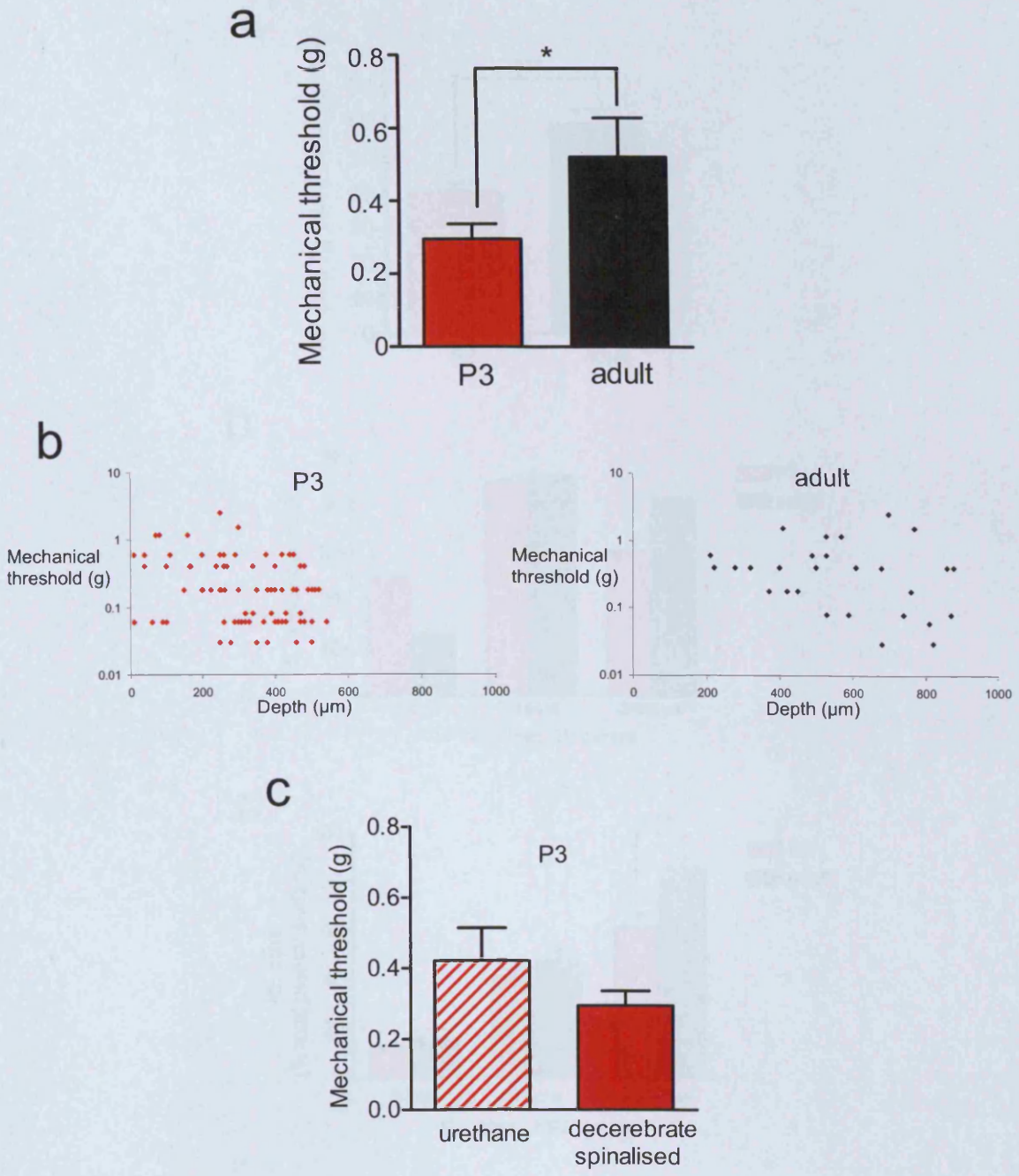


Figure 3.2 Mechanical thresholds increase with postnatal development

Data for the decerebrated, spinalised preparation. (a) Red and black bars show the mean von Frey hair thresholds at postnatal day (P) 3 (n = 87) and in the adult (n = 29) respectively. Error bars represent SEM. (b) There was no correlation between cell depth and von Frey hair threshold at P3 (left) or in the adult (right). (c) Mean mechanical thresholds at P3 are not significantly different in the urethane anaesthetised (striped) and the decerebrate, spinalised preparations.

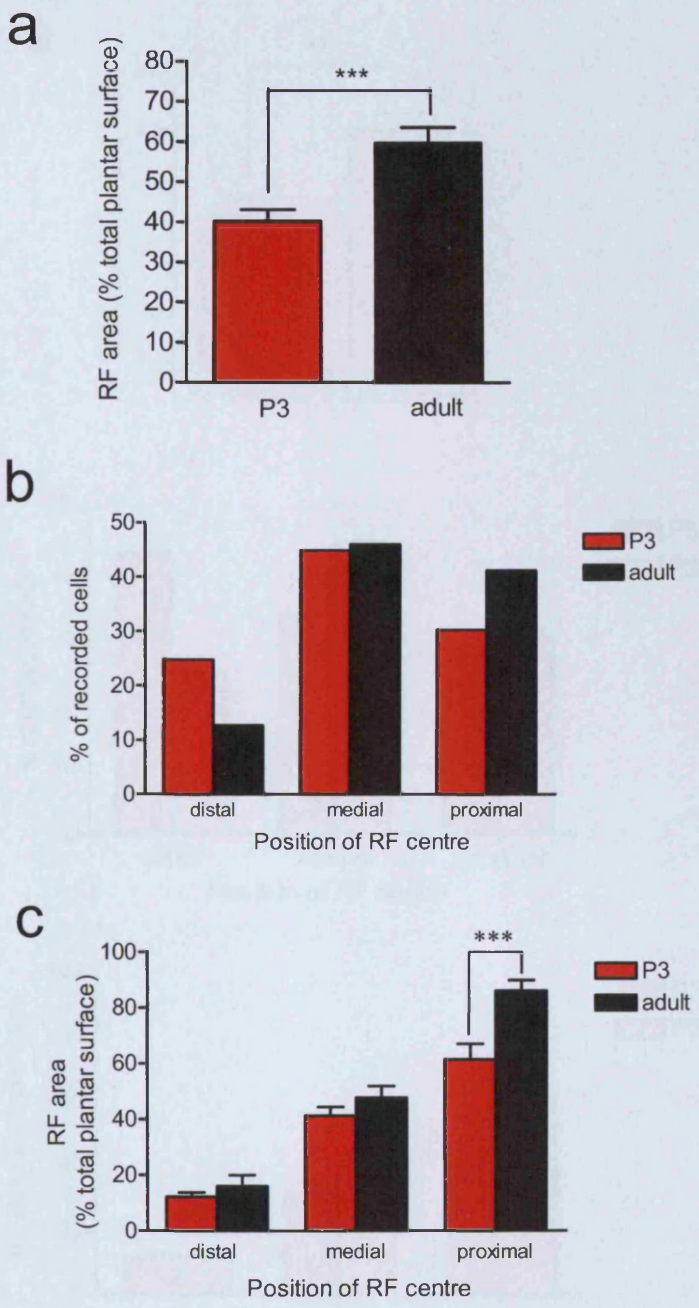


Figure 3.3 Receptive field size increases over postnatal development in the decerebrate, spinalised preparation

(a) Mean receptive field (RF) areas are significantly larger in the adult than at postnatal day (P) 3 in the decerebrate, spinalised preparation ($p = 0.0001$). (b) There was a larger proportion of cells with distal RFs at P3 than in the adult. (c) At both ages, RF size increases as RF centre moves proximally ($p < 0.0001$). A two-way ANOVA showed a significant main effect of age on RF size: RFs are smaller in this preparation at P3 than in the adult ($p < 0.05$). This effect was most pronounced for the cells in the proximal category ($p < 0.001$).

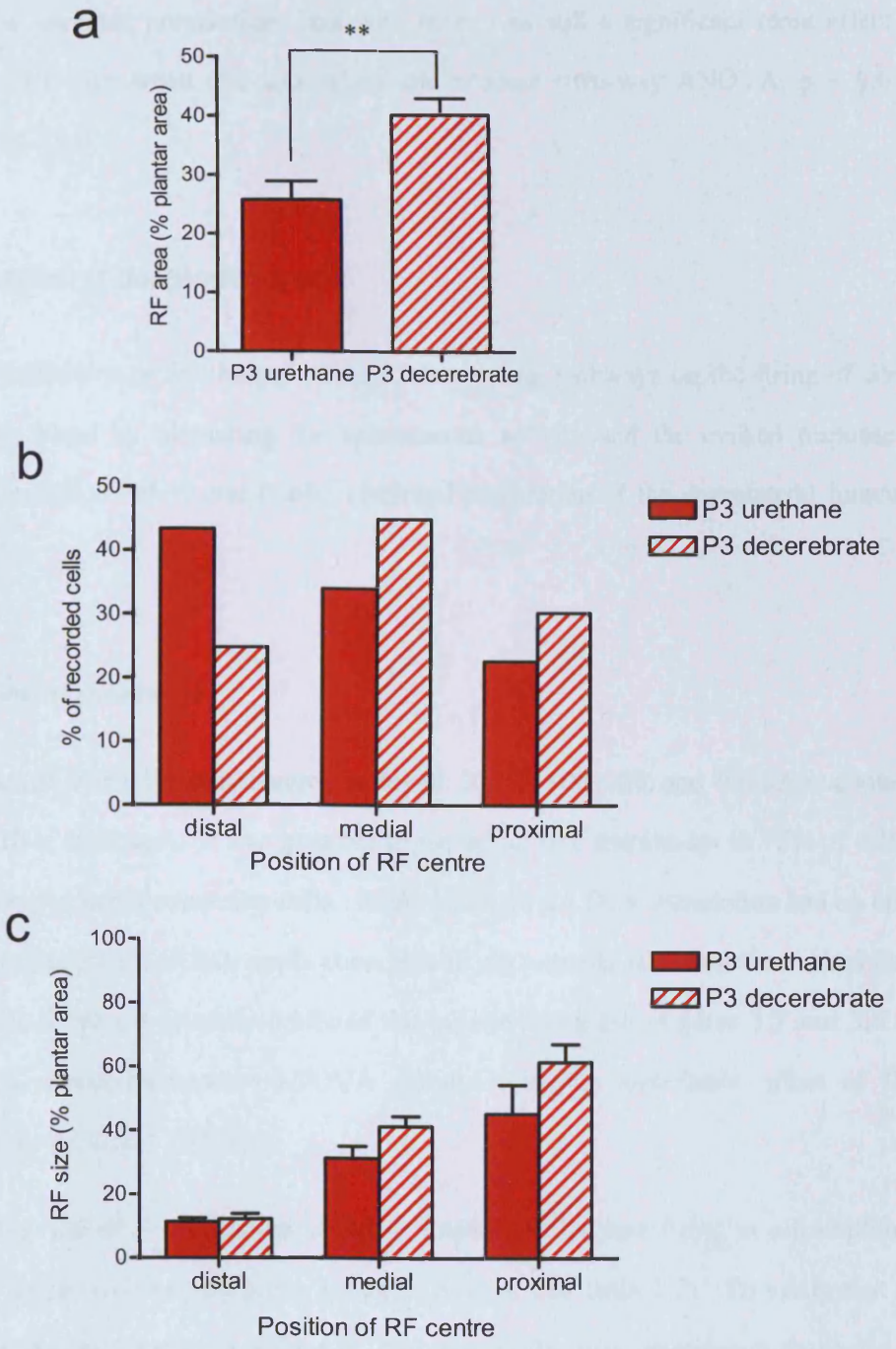


Figure 3.4 Receptive field sizes increase in the decerebrate, spinalised preparation
(a) Receptive field sizes at P3 are larger in the decerebrate, spinalised preparation than in the urethane anaesthetised preparation ($p<0.01$). (b) A larger proportion of distal receptive fields was recorded in the urethane preparation. (c) Receptive field sizes were larger in the decerebrate, spinalised preparation even when plantar region was taken into account (two-way ANOVA, $p = 0.017$).

recorded in the urethane preparation; however, there was still a significant main effect of preparation on RF size when this was taken into account (two-way ANOVA; $p = 0.017$; figures 3.4b and 3.4c).

3.3.2 Stimulation of descending tracts

The potential inhibitory or facilitatory effect of descending pathways on the firing of dorsal horn cells was tested by measuring the spontaneous activity and the evoked response to mechanical stimulation before and during electrical stimulation of the dorsolateral funiculus (DLF).

3.3.2.1 Mechanical stimulation

Five amplitudes of DLF stimulation were employed: 20, 50, 100, 200, and 400 μA in a total of 20 cells ($n = 10$ at each age). It was possible to record all five amplitudes in 75% of cells; a subset was recorded in the remaining cells. In the adult, 20 μA DLF stimulation had no effect on firing to suprathreshold vF hair application, but 50 μA partially inhibited the evoked firing and 100, 200 and 400 μA strongly inhibited the evoked firing (see figures 3.5 and 3.6 and table 3.2). A repeated-measures ANOVA showed a highly significant effect of DLF stimulation in the adult ($p < 0.0001$).

At P3, however, the DLF stimulation failed to inhibit vFh-evoked firing at all amplitudes tested, as can be clearly seen in figures 3.7 and 3.8a (see also table 3.2). To verify that any lack of effect was not due to a failure of the chosen electrical parameters to excite the descending fibres, the dorsal columns were left uncrushed in two additional preparations at P3. In these instances, the electrical stimulation induced a time-locked evoked potential, indicating that the stimulus parameters were indeed capable of activating fibres at this age (see figure 3. 8b).

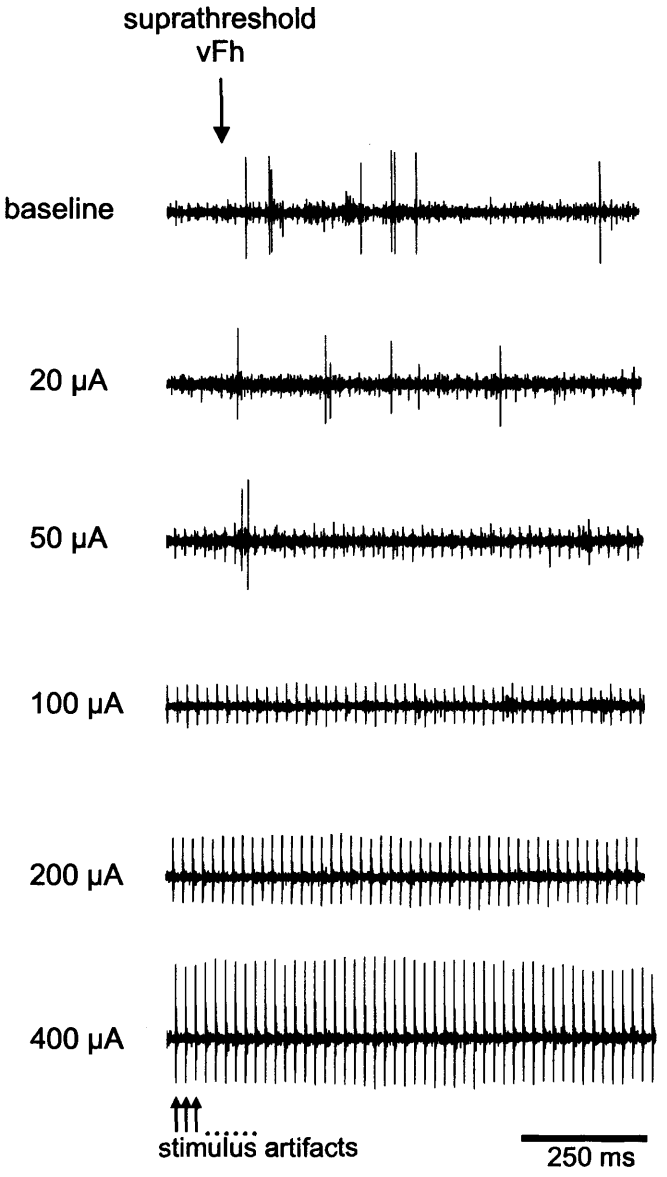


Figure 3.5 Sample traces during stimulation of the dorsolateral funiculus in the adult Sample traces from one adult dorsal horn cell showing the first 1 s of the response to application of a suprathreshold von Frey hair (vFh) during no electrical stimulation (baseline, top) and during electrical stimulation of the dorsolateral funiculus (DLF) at 20, 50, 100, 200, and 400 µA. Evoked firing is partially inhibited during 50 µA DLF stimulation, and fully inhibited during 100, 200 and 400 µA DLF stimulation. Stimulus artifacts can clearly be seen during the periods of stronger stimulation.

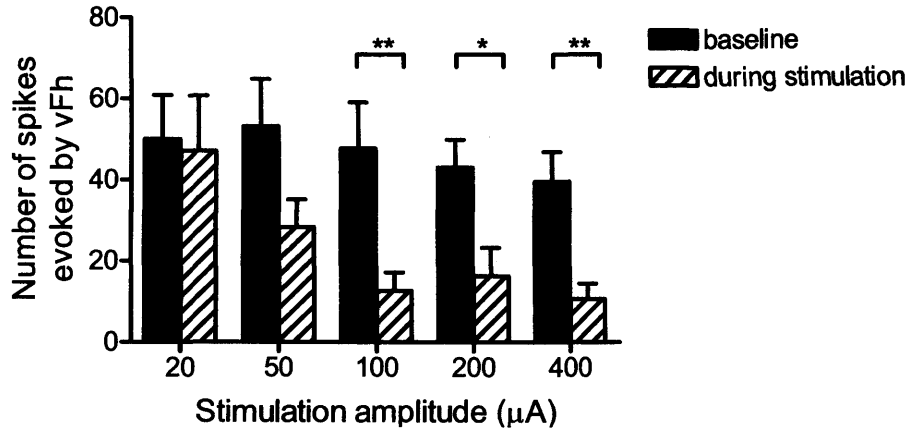


Figure 3.6 Evoked spikes during stimulation of the dorsolateral funiculus in the adult The mean number of spikes fired by adult dorsal horn cells in response to suprathreshold von Frey hair (vFh) application was significantly decreased by electrical stimulation of the dorsolateral funiculus at intensities greater than 100 μA (Two-way ANOVA main effect $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$). Error bars represent SEM.

Stimulation amplitude (μA)	Baseline spikes	Spikes during DLF stimulation	Mean % change	n
ADULT				
20	50.1 +/- 10.9	47.2 +/- 13.6	-9.9 +/- 17.9	6
50	53.3 +/- 11.6	28.3 +/- 6.9	-42.1 +/- 15.7	6
100	47.8 +/- 11.3	12.6 +/- 4.5	-70.1 +/- 13.6	7
200	43.1 +/- 6.8	16.3 +/- 7.0	-64.2 +/- 13.7	8
400	39.7 +/- 7.2	10.7 +/- 3.8	-75.1 +/- 9.3	10
P3				
20	21.5 +/- 4.8	19.6 +/- 4.2	-6.3 +/- 7.3	10
50	21.3 +/- 4.4	21.8 +/- 4.7	2.9 +/- 3.6	10
100	17.7 +/- 3.0	19.7 +/- 3.7	13.9 +/- 12.6	10
200	17.4 +/- 2.8	16.2 +/- 2.8	-7.3 +/- 5.4	9
400	18.8 +/- 3.9	20.9 +/-5.5	9.0 +/- 25.1	9

Table 3.2 Spikes fired to suprathreshold von Frey hair application before and during electrical stimulation of the dorsolateral funiculus (DLF). Percentage change was calculated on a within-cell basis, and the mean of those values is displayed in the table.

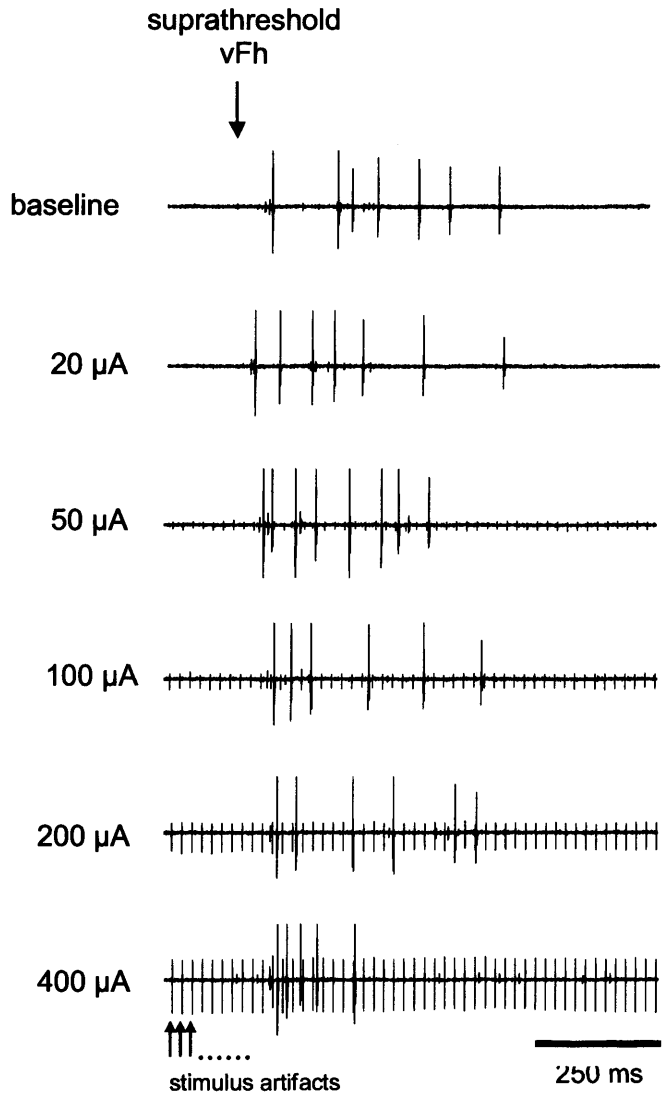


Figure 3.7 Evoked spikes during stimulation of the dorsolateral funiculus at P3
Sample traces from one dorsal horn cell at postnatal day (P) 3 showing the first 1 s of the response to application of a suprathreshold von Frey hair (vFh) during no electrical stimulation (baseline, top) and during electrical stimulation of the dorsolateral funiculus (DLF) at 20, 50, 100, 200, and 400 µA. Evoked firing is not inhibited by the DLF stimulation. Stimulus artifacts can clearly be seen during the periods of stronger stimulation.

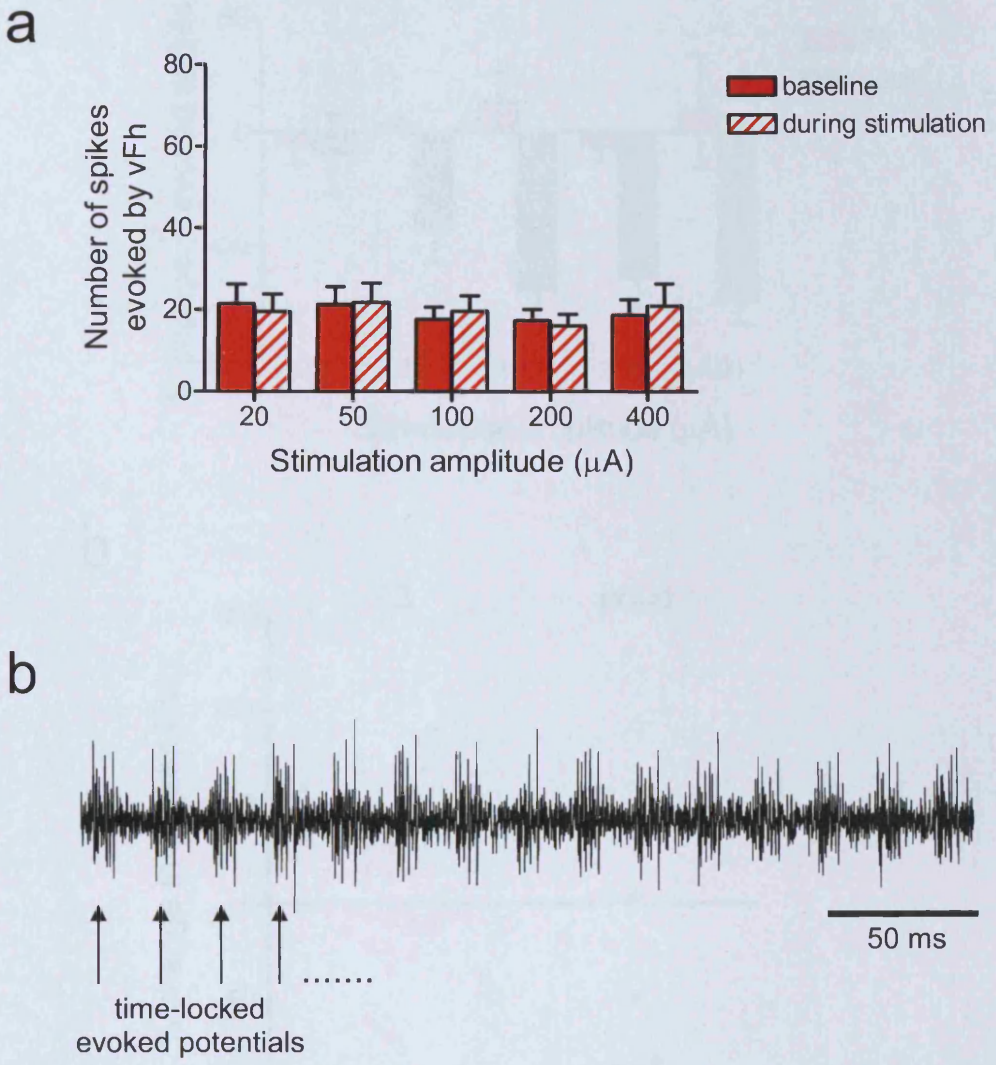


Figure 3.8 Evoked spikes during stimulation of the dorsolateral funiculus at P3

(a) The mean number of spikes fired by dorsal horn cells at postnatal day (P) 3 in response to suprathreshold von Frey hair (vFh) application was unaffected by electrical stimulation of the dorsolateral funiculus (DLF) at all stimulus intensities. Error bars represent SEM. (b) Sample trace from one P3 cell in a preparation with intact dorsal columns. In this preparation, 100 μA DLF stimulation at 50 Hz produced a direct, time-locked response in the dorsal horn, evoked by antidromic activation of the dorsal column fibres.

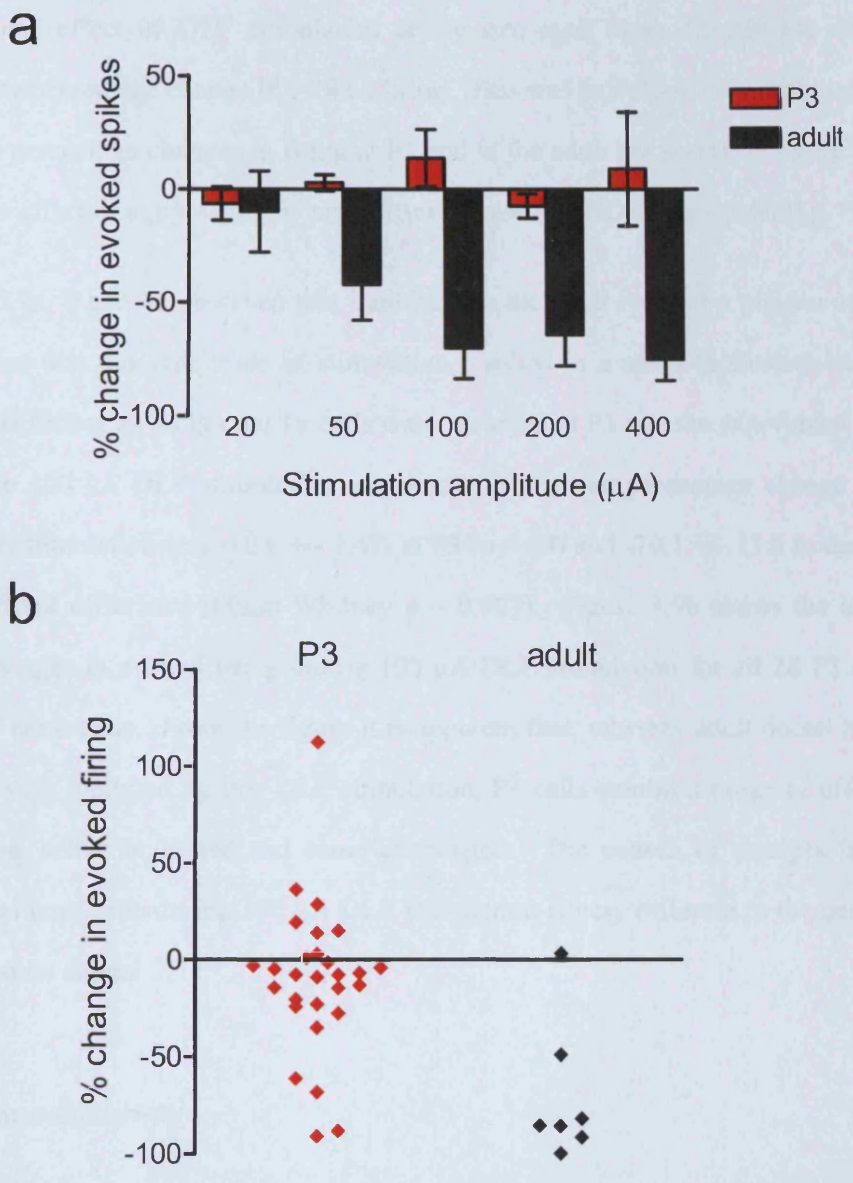


Figure 3.9 Percentage change in evoked firing at both ages

(a) The mean percentage change in firing to suprathreshold von Frey hair application during stimulation of the dorsolateral funiculus (DLF) at a range of amplitudes. There was little change in response for cells at postnatal day (P) 3, but a clear inhibition of firing with DLF stimulation in adult cells. (b) The percentage change in firing to a suprathreshold von Frey hair during 100 μA DLF stimulation for individual cells at P3 and in the adult.

To compare the effect of DLF stimulation at the two ages more directly the data were replotted as the percentage change in evoked firing. This was calculated on a within-cell basis and the mean percentage changes in firing at P3 and in the adult are shown in figure 3.9a and table 3.2. The effect of age was highly significant (two-way ANOVA: $p < 0.0001$).

From figure 3.9a, it can be observed that inhibition in the adult reached a plateau at 100 μA stimulation, but that this amplitude of stimulation resulted in a small facilitation at P3. To investigate this further an additional 18 cells were recorded at P3 and the experiment repeated using only the 100 μA DLF stimulation amplitude. The mean percentage change in firing during 100 μA stimulation was -10.6 +/- 7.4% at P3 (n = 28) and -70.1 +/- 13.6 in the adult (n = 7), a significant difference (Mann Whitney p = 0.007). Figure 3.9b shows the individual percentage changes in evoked firing during 100 μA DLF stimulation for all 28 P3 cells and the original 7 adult cells. From the figure it is apparent that, whereas adult dorsal horn cells are almost always inhibited by this DLF stimulation, P3 cells exhibit a range of effects with some inhibited, some facilitated and some unchanged. The pattern of changes in evoked firing of dorsal horn cells during 100 μA DLF stimulation is very different in the neonatal rat than in the mature animal.

3.3.2.2 Spontaneous activity

A total of 19 cells were found with spontaneous activity (P3: n = 11; adult: n = 8). In the adult, the effect of DLF stimulation on spontaneous activity was also measured at five different stimulation amplitudes: 20, 50, 100, 200, and 400 μA. The results were similar to those observed for evoked firing: 20 μA DLF stimulation had no effect on the rate of spontaneous firing, 50 μA stimulation caused a partial inhibition of firing, and 100, 200 and 400 μA strongly inhibited spontaneous activity (see table 3.3 and figure 3.10). A repeated-measures ANOVA showed that the effect of DLF stimulation was highly significant ($p<0.0001$).

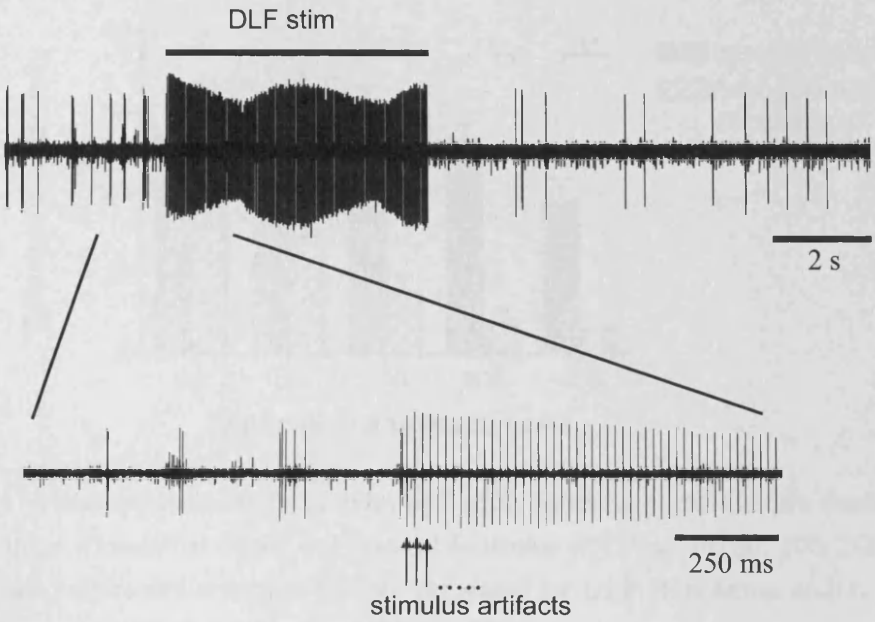


Figure 3.10 Sample trace of spontaneous activity during stimulation of the dorsolateral funiculus in the adult

Sample trace for one adult cell with spontaneous activity. Stimulation of the dorsolateral funiculus (DLF) at 100 μA for 5 s completely inhibited the ongoing activity both during the stimulation and for 2 s afterwards.

At P3, the effect of DLF stimulation on spontaneous activity was measured only at the 100 μA stimulation amplitude. Somewhat surprisingly, given the results for evoked activity described above, DLF stimulation strongly inhibited spontaneous activity in 8/11 (73%) cells tested, similar to the proportion inhibited in the adult at the same stimulation amplitude [6/7 cells (86%)]. The mean spontaneous firing rate at P3 was significantly lower during the 100 μA stimulation (1.5 +/- 0.6 Hz) than during the baseline period (5.2 +/- 1.1 Hz; Wilcoxon $p < 0.005$; see figure 3.13). For comparison between the two ages, the data were recalculated as the percentage change in spontaneous firing (in Hz) during DLF stimulation. The mean percentage change was very similar at the two ages [P3: -68.7 +/- 12.9% (n = 11); adult: -66.9 +/- 10.0 (n = 7)] as was the distribution (see figure 3.14).

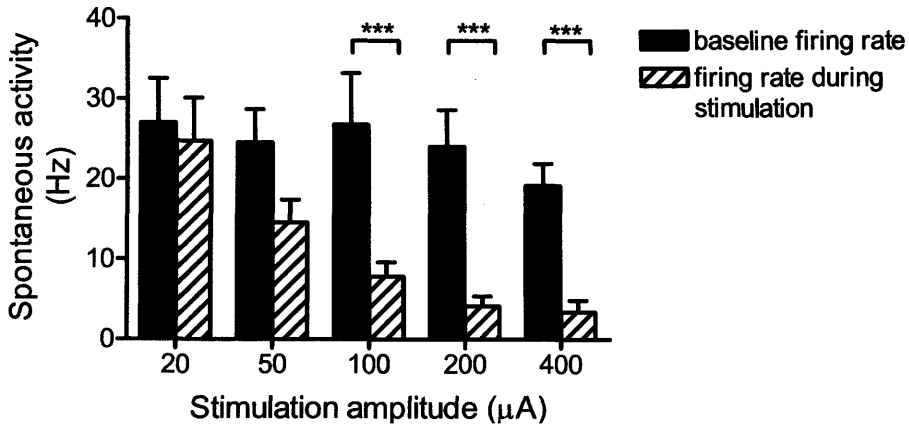


Figure 3.11 Mean spontaneous firing (Hz) in 8 adult dorsal horn cells before (baseline) and during electrical stimulation of the dorsolateral funiculus (DLF) at 20, 50, 100, 200 and 400 μA. Spontaneous activity was significantly decreased by DLF stimulation at 100, 200, and 400 μA (two-way ANOVA $p < 0.0001$; *** $p < 0.001$).

Stimulation amplitude (μA)	Baseline firing rate (Hz)	Firing rate during DLF stimulation (Hz)	Mean % change	n
20	27.0 +/- 5.5	24.8 +/- 5.4	-13.2 +/- 11.1	6
50	24.5 +/- 4.1	14.6 +/- 2.8	-39.2 +/- 11.5	7
100	26.8 +/- 6.4	7.9 +/- 1.7	-66.9 +/- 10.0	7
200	24.0 +/- 4.6	4.1 +/- 1.2	-83.9 +/- 3.9	7
400	19.2 +/- 2.7	3.4 +/- 1.4	-81.5 +/- 7.5	8

Table 3.3 Spontaneous activity (Hz) in the adult before and during electrical stimulation of the dorsolateral funiculus (DLF). Percentage change was calculated on a within-cell basis, and the mean of those values is displayed in the table.

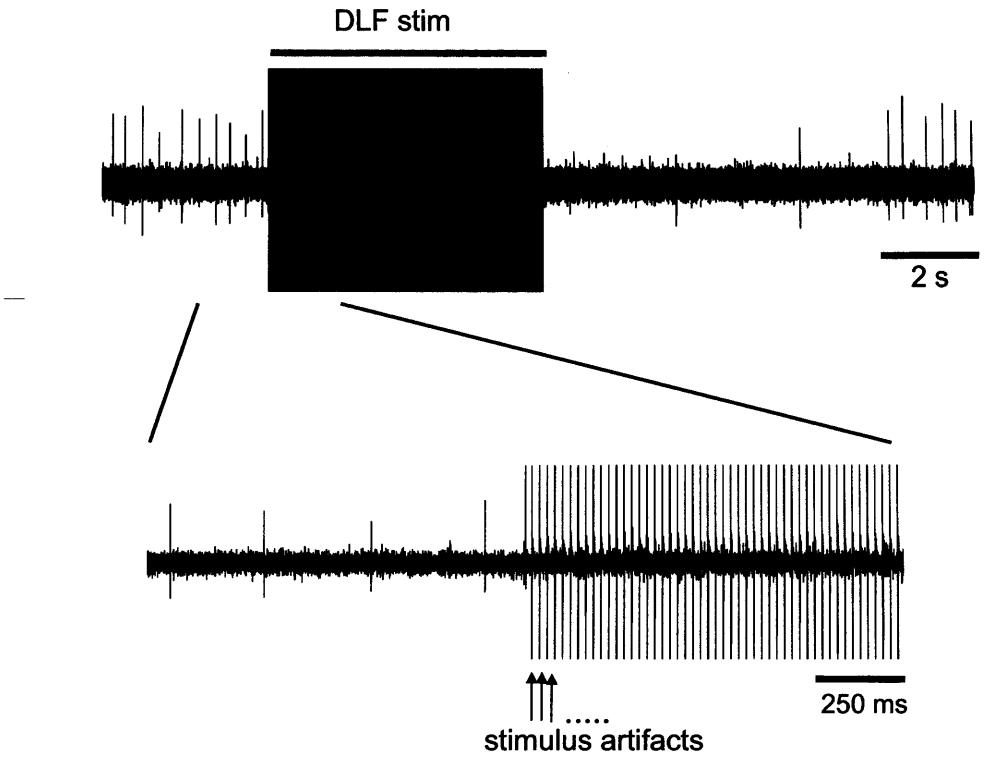


Figure 3.12 Sample trace for one cell at postnatal day (P) 3 with spontaneous activity Stimulation of the dorsolateral funiculus (DLF) at 100 μA for 5 s completely inhibited the ongoing activity both during the stimulation and for 6 s afterwards.

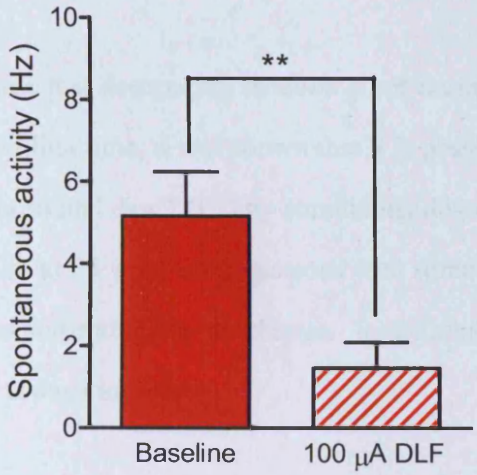


Figure 3.13 Spontaneous activity during stimulation of the dorsolateral funiculus at postnatal day 3

Mean spontaneous firing (Hz) at postnatal day (P) 3 was significantly reduced during 100 μA stimulation of the dorsolateral funiculus (DLF) (** $p < 0.01$; $n = 11$).

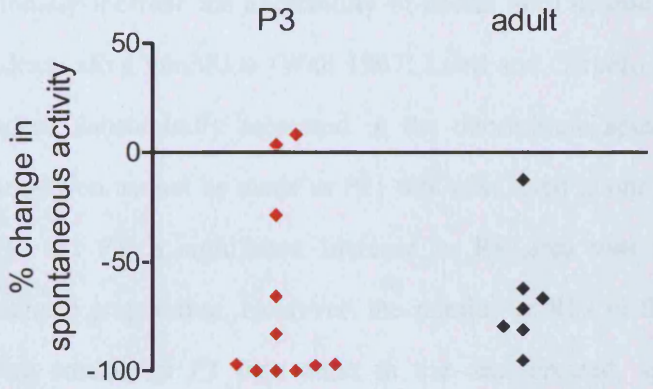


Figure 3.14 Percentage change in spontaneous activity at both ages

The percentage change in spontaneous activity during 100 μA stimulation of the dorsolateral funiculus for individual cells at postnatal day (P) 3 and in the adult.

3.4 Discussion

These results demonstrate that descending modulation of dorsal horn circuitry is immature in the neonatal rat. For the first time, it was shown that it is possible to inhibit the evoked firing of dorsal horn cells at postnatal day 3 (P3) by stimulating descending pathways; however, the effects of this stimulation at P3 were heterogeneous with some cells showing inhibition, some showing facilitation and some showing no change. In the adult, by comparison, the effect of stimulation was almost always inhibitory.

3.4.1 Effect of preparation on dorsal horn cell properties

The preparation used for these experiments involved decerebrating and spinalising the animals, and subsequent removal of anaesthesia. There were therefore two major differences between this and the urethane-anaesthetised preparation used in chapter 2, both of which are likely to impact on dorsal horn cell responses. First, removal of anaesthesia would be expected to increase the responsiveness of recorded cells at both ages. Second, the spinalisation would be expected to additionally increase the excitability of dorsal horn neurons in the adult through removal of tonic descending inhibition (Wall 1967; Laird and Cervero 1990). The receptive field size was indeed substantially increased in the decerebrate spinalised mature animal (though a true comparison cannot be made as P21 rats were used in one preparation and ~P42 rats in the other). At P3, a significant increase in RF area was also observed in the decerebrated, spinalised preparation. However, the relation to RFs in the mature animal was reversed: RFs were smaller at P3 than adult in the decerebrated, spinalised preparation, whereas they were larger at P3 in the urethane-anaesthetised preparations (see section 2.3.1.2). This suggests that the effects of spinalisation are different at P3 than in the adult, though from these data it is not possible to deduce whether this is due to merely a lack of descending inhibition at P3, or to removal of descending facilitation at P3. A straight comparison of RFs in the spinalised versus the intact rat found that the procedure causes a reduction in receptive

field (RF) area at P7 (D. Ririe and M. Fitzgerald, personal communication), consistent with the hypothesis of tonic descending faciliation in neonates (Hathway et al. 2006).

Another major change in this chapter from the preparation used in chapter 2 was P42 rats were used instead of P21 rats. This was because during pilot studies it was found that P42 rats were better able to tolerate the decerebration than P21 rats. In the latter, excessive bleeding often led to early termination of the experiment.

3.4.2 Stimulation parameters

The dorsolateral funiculus (DLF) is a major output pathway from the brainstem to the dorsal horn and contains axons from nuclei of the rostroventral medulla (RVM) and the dorsal reticular nucleus, as well as from other sites in the mesencephalon and diencephalon. Although care was taken to position the stimulating electrodes in the DLF, it is possible that other descending tracts such as the VLF may have been recruited, especially at the highest stimulus amplitudes used. The stimulation paradigm used in these experiments was therefore not designed to test the role of any one particular descending pathway, but was used to assess the overall effect of descending controls on dorsal horn cell firing through postnatal development. It would be of great interest to record from neonatal dorsal horn cells whilst stimulating directly in, for example, the RVM, especially as it has been reported that different stimulus amplitudes can differentially recruit populations of 'on' and 'off' cells in this structure (Zhuo and Gebhart 1997). However, such an experiment would be technically demanding to perform in the neonatal rat pup.

3.4.3 Postnatal development of descending modulation

The present results in general support previous findings of a deficit in descending inhibition in the immature rat spinal cord (Fitzgerald and Koltzenburg 1986; van Praag and Frenk 1991; Boucher et al. 1998). The inhibition of mechanically evoked firing by DLF stimulation was

significantly less at P3 than in the adult. However, when a larger population of cells was studied, it became apparent that activation of descending tracts can sometimes provoke both inhibition and facilitation of firing at P3, as well as often being without effect. These results are the first direct demonstration of this phenomenon in the neonate. Interestingly, the DLF stimulation inhibited spontaneous activity to a similar degree at both ages. One possible explanation for this is that the descending inhibitory connections may be present but weak at P3, so that they can often inhibit ongoing network activity but are rarely strong enough to inhibit the excitatory drive that accompanies an evoked stimulus. This would be consistent with the absence of descending inhibition of strong electrically evoked C-fibre activity at early postnatal ages (Fitzgerald and Koltzenburg 1986). It is worth noting that the baseline spontaneous activity observed at P3 is in the region of 5 Hz, whereas it is frequently greater than 20 Hz in adult cells. Thus, weaker descending inhibition may be sufficient to inhibit this low rate of firing at P3 to a similar degree as the strong descending inhibition inhibits the higher rate of firing in the adult. Nevertheless, the end result is still a silencing of firing in a number of cells in the neonatal dorsal horn, which presumably is of functional significance.

The deficit in descending inhibition of evoked responses in the neonate may be linked to the weak and asynchronous C fibre input to the dorsal horn during the early postnatal period (Jennings and Fitzgerald 1998; Baccei et al. 2003). Systemically applied capsaicin destroys a large proportion of the C fibre afferents, and rats treated in this way at birth display a clear deficit in tonic descending inhibition as adults (Cervero and Plenderleith 1985). This indicates that the maturation of descending inhibition is dependent on normal development of the C fibre input, which occurs over the first two postnatal weeks. The presence of powerful descending controls prior to maturation of the strong excitatory input mediated by C fibres could lead to excessive dampening of dorsal horn responses in the neonate; hence a dependence of descending inhibition on the maturation of the C fibre input could be adaptive. There is strong evidence that some supraspinal modulatory systems are activated only by noxious peripheral stimulation (Villaneuva and LeBars 1995), and some are now known to be even more selective, being preferentially activated by C-fibre nociceptors over A-fibre

nociceptors (Lumb et al. 2002). Conversely, several supraspinal structures are known to exert a descending effect that selectively inhibits dorsal horn responses to noxious stimuli (Fleetwood-Walker et al. 1988; Waters and Lumb 1997; Workman and Lumb 1997; Hudson et al. 2000), and recent work has extended this by proposing a differential effect of PAG activation on dorsal horn responses to A- and C- nociceptor stimulation (McMullan and Lumb 2006a,b). The links between descending modulatory centres and afferent input therefore appear to be both bidirectional and selective.

3.4.4 Conclusions

The present results demonstrate that descending modulation in the neonatal rat pup is immature: electrical stimulation of the dorsolateral funiculus had heterogenous effects on dorsal horn responses to suprathreshold stimuli. Evoked firing was inhibited in some cells, but facilitated or unaffected in most cells. In contrast, the same stimulation in the adult produced clear inhibition of evoked firing. Given the importance of tonic descending inhibition in shaping the receptive fields and response properties of adult dorsal horn neurons and nociceptive reflexes, it seems likely that the immaturity in descending modulation in the neonate contributes to the differences in nociceptive reflexes and behaviour observed at this age.

Chapter 4

Postnatal development of inhibitory cutaneous receptive fields

4.1 Introduction

Tactile and nociceptive circuits show increased excitability during the early postnatal period, suggesting a possible deficit in inhibitory processing in the dorsal horn. Any such deficit must be more subtle than a mere lack of inhibitory control because GABAergic inhibition has been shown to be functional in the neonatal rat dorsal horn (see chapter 2). Maturation of descending controls clearly plays an important role in the postnatal development of the nociceptive system (see chapter 3). Returning to the segmental level, however, it is possible that although inhibition may be relatively mature at the synapse in the neonate, the network-level wiring of inhibitory circuits may be undergoing modification.

There have been reports of postnatal alterations in inhibitory connections across a range of sensory modalities. For example, developmental frequency-tuning of neurons in the auditory brainstem requires refinement of inhibitory connections, including both functional weakening and structural elimination of synapses (Kandler and Gillespie 2005). In the gerbil, inhibitory terminals from neurons of the medial nucleus of the trapezoid body (MNTB) initially occupy a broad portion of the tonotopic map in the lateral superior olive (LSO) but are pruned back to a narrow region during the second and third postnatal weeks, concomitant with a reduction in the dendritic spread of LSO neurons (Sanes and Siverls 1991; Sanes et al 1992). Importantly, the postnatal development of precise tonotopy is dependent on auditory experience: removal of inputs from the cochlea to the MNTB prevented the refinement of tuning in the LSO (Sanes and Takacs 1993). Activity is also thought to play a vital role in the tuning of neuronal responses in the somatosensory system, where the arrangement of both excitatory and inhibitory synapses in whisker barrel cortex is disrupted by sensory deprivation during development (Fox and Wong 2005). In the visual system, patch-clamp recording of neurons in the optic tectum of *Xenopus* tadpoles has shown that there is a gradual refinement and matching of excitatory and inhibitory visual receptive fields (RFs) during development (Tao and Poo, 2005). At early stages in tadpole development, excitatory and inhibitory RFs are large and uncorrelated with each other: the excitatory and inhibitory RFs for a single cell are

frequently centred on different regions of visual space. During successive developmental stages, the excitatory and inhibitory RFs decrease in size and the RF centres gradually become more aligned until they have closely matching spatial profiles (Tao and Poo, 2005).

It is likely that this drive towards balancing of excitatory and inhibitory inputs during development is an underlying wiring principle in the central nervous system, and a similar refinement of RFs may occur in the dorsal horn during the postnatal period. Indeed, a comparable reduction in the size of excitatory cutaneous RFs over postnatal development has already been well documented (Fitzgerald, 1985; Torsney and Fitzgerald, 2002) and was also observed in experiments described in chapter 2 of this thesis. However, postnatal changes in the size of inhibitory RFs of dorsal horn cells and in the spatial relationship between inhibitory RFs and their excitatory counterparts remain to be examined.

4.1.1 Inhibitory receptive fields of adult dorsal horn cells

The development of inhibitory circuitry in the dorsal horn can be investigated by mapping inhibitory receptive fields (RFs). Several different forms of inhibitory RF have been described in the adult rat dorsal horn, some dependent on descending influences and others with a segmental mechanism.

4.1.1.1 Ipsilateral surround inhibition

Ipsilateral inhibition in the cat can be mediated by large afferent fibres and produced by brushing or applying other light mechanical stimulation within the inhibitory RF (see Willis and Coggeshall 1991). In the rat, ipsilateral noxious stimulation can also inhibit cell firing (for example, Waters and Lumb 2007). The original studies in the cat recorded extracellular spikes *in vivo* and described the inhibitory RFs as forming a "surround" to the excitatory RF (Hillman and Wall, 1969). More recently, *in vivo* patch clamping in the adult rat dorsal horn has become possible, enabling the study of subthreshold excitatory and inhibitory events in

the intact animal. Using this technique in urethane-anaesthetised adult rats it has been shown that, for the majority of cells recorded in substantia gelatinosa, IPSCs can be provoked by brush and innocuous touch of the ipsilateral hind limb (Narikawa et al, 2000). The IPSCs recorded *in vivo* were blocked by strychnine and bicuculline, indicating that they are mediated by GlyRs and $GABA_A$Rs, as would be anticipated from *in vitro* studies. The authors also attempted to map the inhibitory and excitatory RFs and found that both RFs were large, covering the lower leg, upper leg and thigh. Moreover, they reported little difference in RF size between inhibitory and excitatory RFs, which at first appears inconsistent with the data from extracellular recordings that suggests a centre – surround organisation. However, Narikawa et al (2000) did not study the receptive fields in detail and it is likely that the relative strength and frequency of IPSCs and EPSCs varied considerably throughout these overall RFs. These large RFs for subthreshold events could therefore still produce a centre – surround RF for suprathreshold action potentials.

This centre and antagonistic surround organisation of receptive fields is common in the early processing stages of other sensory pathways. Retinal ganglion cells, cells in the lateral geniculate nucleus, and simple cells in cortical area V1 have this arrangement, as do neurons in the auditory midbrain (HUBEL and WIESEL, 1961; Knudsen and Konishi, 1978). In these other sensory modalities, one role for the centre – surround organisation is to enhance the contrast of the signal relative to the noise, enabling more accurate extraction of visual features and identification of tones. It is possible that cutaneous surround inhibitory fields in the dorsal horn have a similar role in enhancing touch discrimination, but this is unlikely as touch discrimination is thought to be mediated via the dorsal column pathway, which bypasses the dorsal horn (Azulay and Schwartz, 1975; Dobry and Casey, 1972).

An alternative role for this ipsilateral inhibition was proposed by Melzack and Wall in their influential gate control theory (Melzack and Wall, 1965), whereby activity in large myelinated fibres would, via an inhibitory interneuron, close the "gate" and limit the response of nociceptive output cells until the inhibition was relieved and the gate reopened by increased

input from small afferent fibres. Such a system would serve to filter the signal from the periphery so that the nociceptive output cells would only be activated when the balance of the stimulation was predominantly nociceptive, rather than responding to an overall increase in all cutaneous input. Although the theory also included a role for higher centres in controlling the sensitivity of the gate, the crucial large-fibre mediated inhibition was proposed to be a purely spinal mechanism. The low-threshold surround inhibition observed in the dorsal horn has a segmental mechanism as it remains after spinalisation, and so fits well into the gate control theory. Several aspects of the original gate control theory have since been criticised (Nathan, 1976), but the hypothesis that inhibition evoked by activity in large fibres may serve to dampen responses to noxious stimulation remains important.

4.1.1.2 Diffuse noxious inhibitory controls

A contrasting form of inhibition is observed in wide dynamic range neurons in intact anaesthetised rats: noxious stimulation (usually pinch or noxious heat) applied outside the excitatory RF causes a strong and prolonged inhibition of neuronal firing lasting for many minutes (Gebhart et al, 1981; Le Bars D. et al, 1979a; Ness and Gebhart, 1991a; Ness and Gebhart, 1991b). The RFs for this type of inhibition spread both ipsilaterally and contralaterally and are extremely large, often covering the entire animal. This heterotopic inhibition of neuronal activity is completely or largely abolished by spinalisation, and the strong descending component is termed diffuse noxious inhibitory controls (DNIC) because of the large and diffuse RFs and the modality-specific stimulation required to provoke it (Le Bars D. et al, 1979b; Villanueva et al, 1986). It has been suggested that DNIC may serve to enhance the contrast in firing between those WDR cells activated by the noxious stimulus and the remaining WDR population, which will be inhibited by the same stimulus (see Willis and Coggeshall 1991).

4.1.1.3 Segmental contralateral inhibition

In some preparations, spinalisation removes all heterotopic inhibition (Le Bars 1979b); however, in other studies some inhibition has been observed to remain even after removal of DNIC by spinalisation (Cadden et al, 1983; Fitzgerald, 1982; McGaraughty and Henry, 1997; Sandkuhler et al, 1993; Weng and Schouenborg, 1996). The discrepancy between the types of inhibition revealed in these experiments is almost certainly due to differences in the type and depth of anaesthesia employed (Tomlinson et al, 1983) and also perhaps in the particular noxious stimulation used to produce the inhibition. For example some studies use pinch, which is usually a short and very localised noxious insult, whereas others use immersion of a hind paw in hot water, which is a more prolonged stimulus acting on a large cutaneous area (McGaraughty and Henry, 1997).

Cutaneous inhibitory RFs of withdrawal reflexes can be accurately mapped using electromyography. In animals spinalised at thoracic levels, the inhibitory RFs have been found to have both an ipsilateral and a contralateral component. The contralateral inhibitory RFs were smaller and weaker, but otherwise were mirror images of the ipsilateral inhibitory RF (Weng and Schouenborg, 1996). In extracellular recordings from dorsal horn neurons, the segmental inhibition reported after spinalisation is much weaker than that seen in intact animals, lasting for seconds rather than minutes, and the inhibitory RFs are also much smaller than those mediated by DNIC, often restricted to parts of the hind paw and tail. Furthermore, for individual cells, the inhibitory RF on the contralateral paw is commonly the mirror image of the excitatory RF on the ipsilateral paw (Fitzgerald, 1982). Unlike the low-threshold ipsilateral inhibitory RFs described above, the segmental contralateral inhibition is produced only by noxious cutaneous stimulation, or by stimulation of the contralateral sciatic nerve at intensities sufficient to recruit C fibres. It has also been reported that this inhibition is more frequently observed in cells from deep laminae than those from superficial laminae (Fitzgerald, 1982; Fitzgerald, 1983), where a greater proportion of cells are excited by contralateral stimulation.

There are two major anatomical explanations for the presence of contralateral inhibition: first, that primary afferents project contralaterally; or second, that there is a polysynaptic commissural pathway connecting the two sides of the dorsal horn. Direct projections of primary afferent fibres across the midline do exist in the spinal cord but are more apparent in thoracic and sacral segments than at lumbar levels, where there are very few (Chung and Coggeshall, 1983; Culberson et al, 1979; Light and Perl, 1979), so this is an unlikely source of contralateral effects. In contrast, anatomical studies have shown that the two sides of the spinal cord are well connected by commissural fibres, for which one key role is to coordinate left – right locomotion (Kiehn, 2006). Moreover, a dorsal commissural system at lumbar levels has been described, with many of the fibres originating and terminating in the dorsal horn, particularly in lamina II (Rethelyi 1973). It has been proposed that there may be an excitatory contralateral input to neurons in lamina II, which then inhibit cells in the deeper laminae (Fitzgerald, 1983). This is consistent with the data, but remains a hypothesis.

4.1.2 Inhibitory receptive fields in the neonatal dorsal horn

Little work has been conducted on inhibitory cutaneous RFs in the neonate. The existence of ipsilateral and contralateral inhibitory RFs from P0 *in vivo* has been noted in passing (Fitzgerald, 1985) but there have been no thorough studies of inhibitory RF organisation. Shay and Hochman (2002) used an ex vivo preparation at P8-10 to make patch-clamp recordings of cells and measure the IPSPs and EPSPs produced by stimulating different dorsal roots. This enabled them to gain a crude estimate of the excitatory and inhibitory RFs for each cell, measured as strength of IPSP or EPSP at each root location (numbered with distance from recorded cell). They found that, at this age, a subset of cells had an inhibitory RF which extended across 3 roots (the maximum tested), with inhibitory strength tending to increase as root distance from the excitation increased. Furthermore, this inhibition was modulated by 5-HT. A second subgroup of cells had no inhibitory input and were not affected by 5-HT (Shay and Hochman, 2002). This study provides some useful information about the organisation of

inhibitory RFs in the neonate; however, experiments were only conducted at one age and so no comparisons with the adult can be drawn.

4.1.3 Aims of the chapter

The overall aim of this chapter was to use the phenomenon of segmental contralateral inhibition as a model to investigate the postnatal development of inhibitory RFs in the rat dorsal horn *in vivo*. More specifically, the aims were:

1) To confirm the presence of contralateral inhibition in the adult rat and to assess the size and location of inhibitory RFs relative to the ipsilateral excitatory RFs.

2) To investigate whether segmental contralateral inhibition is present or absent in the dorsal horn at P3. This has been reported only once, briefly and with few details, so it was important to verify whether the inhibition is present with the same regularity in the neonate as in the adult. The ability of innocuous brush and noxious pinch to inhibit evoked firing was assessed at both ages.

3) If contralateral inhibition is present at P3, to map the inhibitory RFs and to compare the spatial organisation of these with those found in the adult, to test the hypothesis that cutaneous inhibitory RFs become increasingly spatially tuned over postnatal development.

4.2 Materials and methods

4.2.1 Decerebrated, spinalised preparation

P3 and adult (~180 g) Sprague Dawley rats were used in these experiments. Animals were decerebrated as described in section 3.2.1. The spinal cord was exposed at the thoracic level and sectioned to remove all descending influences and to completely isolate the lumbar spinal cord. A period of at least 90 minutes was left between spinalisation and recording, to allow for recovery from spinal shock, and the electrocardiogram was monitored throughout the experiment. A second laminectomy was performed over L4 – L5 to allow extracellular recordings from lumbar dorsal horn neurons, which were carried out as described in section 2.2.2.2. The recording depth from the surface was noted and cells classified as superficial (presumed laminae I and II; depth $\leq$ 200 μm from the dorsal surface at P3, $\leq$ 350 μm in the adult) or deep (presumed laminae III, IV, V, VI). The superficial – deep boundary values were obtained from Nissl stained lumbar sections at each age (Torsney and Fitzgerald 2002). Recorded cells were classified as low threshold (LT), noxious specific (NS) or wide dynamic range (WDR).

4.2.2 Excitatory and inhibitory receptive field analysis

Ipsilateral excitatory receptive fields were mapped out for each cell as described previously and a small clip was then applied to the centre of the receptive field to induce long trains of firing. The contralateral plantar hind paw surface was divided into 34 sub-regions (see figure 4.1) and a 3 s pinch was systematically applied to each region to reveal which contralateral areas inhibited the train of spikes. The order in which the different contralateral plantar regions were pinched was varied from cell to cell to prevent bias. The strength of the inhibition produced by each contralateral pinch was quantified both relative to the baseline firing rate, and in absolute terms. In some cells, the response to gentle brush of the contralateral hindpaw with a cotton bud was also assessed.

4.2.2.1 Relative inhibition

The baseline firing rate in Hz (*B*) was established during the 2 s immediately prior to the contralateral pinch. For each cell, the maximal inhibition was measured as follows: the total number of spikes inhibited by the pinch (*N*) was calculated up to the point at which the cell resumed firing at greater than or equal to the baseline firing rate, regardless of whether this was before or after the termination of the 3 s pinch (see figure 4.2). The duration of inhibition was labelled *t*, and inhibitory strength (*I*) was calculated as:

$$I = (N/Bt) * 100$$

The lesser inhibition produced by pinches to other plantar sub-regions was calculated in the same way, but the value of *t* was kept constant within each cell to allow for comparisons between plantar sub-regions.

4.2.2.2 Absolute inhibition

It might be argued that assessing the strength of inhibition relative to baseline firing produces a bias towards cells with lower initial frequencies of firing. For example, a contralateral pinch which resulted in 10 spikes being inhibited would be classed as much less inhibitory if the baseline firing frequency was 20 Hz than if it was 5 Hz. The strength of the inhibition produced by contralateral pinch was therefore also quantified using the absolute number of spikes inhibited (*N*) as the measure of inhibition. When the inhibitory RFs were remapped using this measure of inhibition, the spatial arrangement of the RFs was very similar to that seen using relative inhibition (see figure 4.3). Subsequent analyses therefore used the relative measure only.

4.2.2.3 Quantitative spatial analysis of inhibitory RFs

Comparisons of inhibitory strength in different plantar regions were conducted on a within-cell basis. For each cell, the contralateral paw area that produced the maximal inhibition when pinched was established. The inhibition from the remaining hind paw areas was

Figure 4.1 Subdivisions of the plantar surface
For mapping of contralateral inhibitory receptive fields, the plantar surface was subdivided into 34 regions.

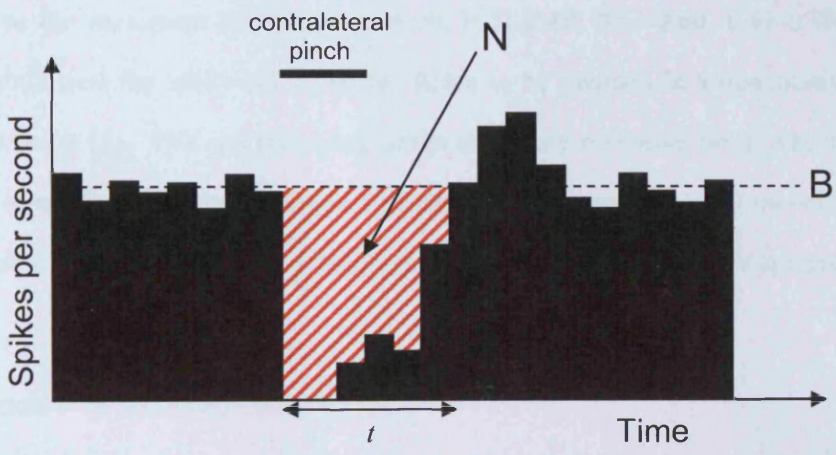


Figure 4.2 Diagram illustrating how maximal inhibitory strength was measured
Maximal relative inhibition was calculated as the total number of spikes inhibited (N; red striped region) relative to the baseline firing rate and the duration of inhibition (B*t*). The value of N alone was taken as a measure of absolute inhibition.

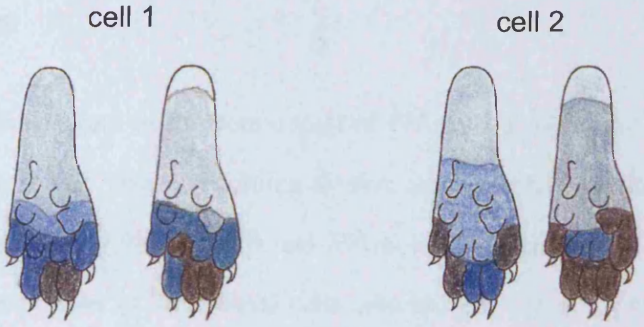


Figure 4.3 Similar spatial maps produced by relative and absolute measures of inhibition

Two sample cells showing similar arrangement of inhibitory receptive fields whether mapped using the relative measure of inhibition (left map of each pair) or the absolute measure of inhibition (right map of each pair).

normalised to the maximum in bins (0.00-0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80, and 0.81-1.00). This allowed the inhibitory receptive fields to be mapped in a quantitative way (see figures 4.10 and 4.11). The spatial tuning of the inhibitory receptive fields was measured by plotting the strength of inhibition against cumulative plantar coverage and measuring the area under the curve for each cell; figure 4.14 illustrates this for a cell from each age group.

4.2.2.4 Alignment of excitatory and inhibitory RFs

To quantify the alignment between ipsilateral excitatory RFs and contralateral inhibitory RFs, the centre of each excitatory RF was identified by eye. This was defined as the spatial centre of the most sensitive region of the RF. The distance between this position and the centre of the region of maximal (0.81-1.00 bin) inhibition was measured for each cell. These measurements were made on the same standardised plantar paw diagrams at each age.

4.3 Results

Single unit recordings were made from a total of 115 cells at two ages: postnatal day (P) 3 (n = 59) and adult (n = 56). Mean recording depths, as measured from the surface of the white matter, were 311.6 +/- 17.9 μm at P3 and 581.6 +/- 23.5 μm in the adult (figure 4.4). A slightly higher proportion of superficial cells was recorded at P3 (18.6%) than in the adult (10.7%).

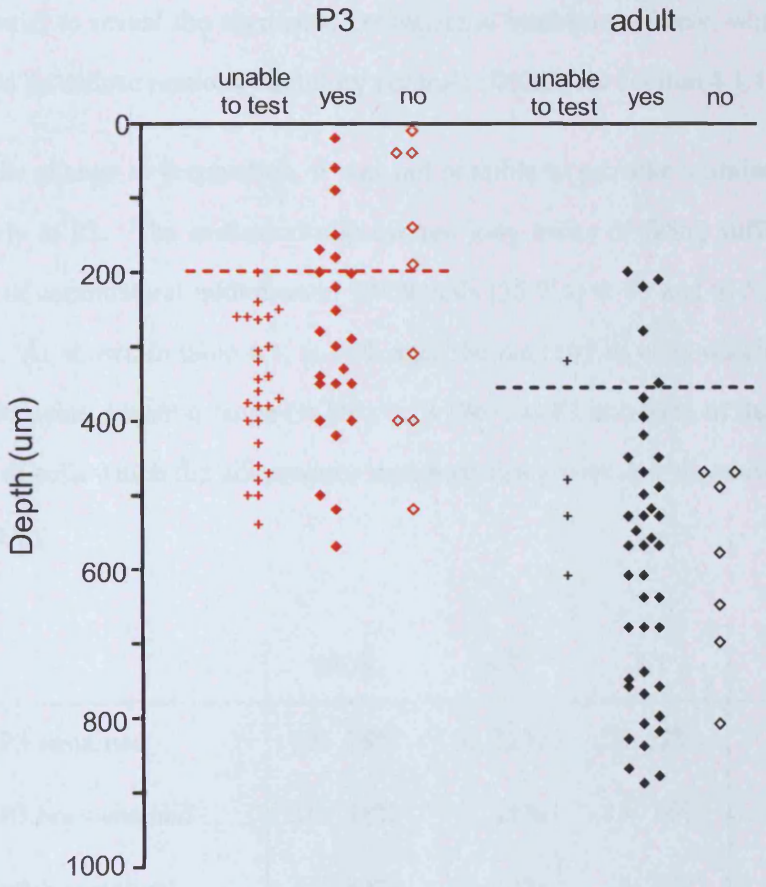


Figure 4.4 Depths of cells recorded for contralateral testing
Cells recorded at P3 (red) and adult (black). Crosses indicate cells which did not produce sustained firing to ipsilateral stimulation and hence could not be tested for contralateral inhibition. Diamonds indicate cells which were tested for inhibition to contralateral pinch: filled symbols represent cells which were inhibited ('yes') and open symbols represent cells which were not inhibited ('no') by this stimulation.

4.3.1 Baseline firing frequency to ipsilateral stimulation

It was difficult to provoke the long spike trains necessary for this experiment with urethane-anaesthetised animals because the deep anaesthesia prevents the majority of cells from firing in a sustained fashion. A decerebrated, spinalised preparation was therefore used (Fitzgerald, 1982) as this allows withdrawal of anaesthesia after the decerebration. The spinalisation also contributed to increased dorsal horn cell excitability in adults at least by removing tonic descending inhibitory inputs (see chapter 3). In addition, removal of these descending inputs was essential to reveal the segmental contralateral inhibitory effects, which would otherwise be masked by diffuse noxious inhibitory controls (DNIC; see section 4.1.1.2).

Despite the change in preparation, it was not possible to provoke sustained firing in all cells, particularly at P3. The ipsilateral clip evoked long trains of firing sufficient to test for the presence of contralateral inhibition in 33/59 cells (55.9%) at P3 and in 52/56 cells (92.9%) in the adult. As shown in table 4.1, at both ages the majority of cells which produced sustained firing were wide dynamic range (WDR) cells (76% at P3 and 83% in the adult), whereas the majority of cells which did not produce sustained firing were low threshold (LT) cells (50% at both ages).

	WDR	NS	LT	total
P3 sustained	25: 76%	4: 12%	3: 12%	33
P3 not sustained	10: 38%	3: 12%	13: 50%	26
adult sustained	43: 83%	6: 12%	3: 5%	52
adult not sustained	1: 25%	1: 25%	2: 50%	4

Table 4.1 Numbers and percentages of cells recorded in each class.
WDR - wide dynamic range; NS - noxious specific; LT - low threshold

Of the cells which produced sustained firing sufficient for contralateral testing, 33.3% (11/33) were classed as superficial at P3, compared with 9.6% (5/52) in the adult (see figure 4.4). For those cells which did produce sustained firing, the mean baseline firing rate to the ipsilateral clip was 35.6 ± 3.9 Hz in the adult but only 5.3 ± 0.5 Hz at P3 (Student's t test $p < 0.0001$, see figure 4.5). Although the spike trains provoked at P3 were much slower than in the adult, they were still sufficiently prolonged to allow adequate experimental testing.

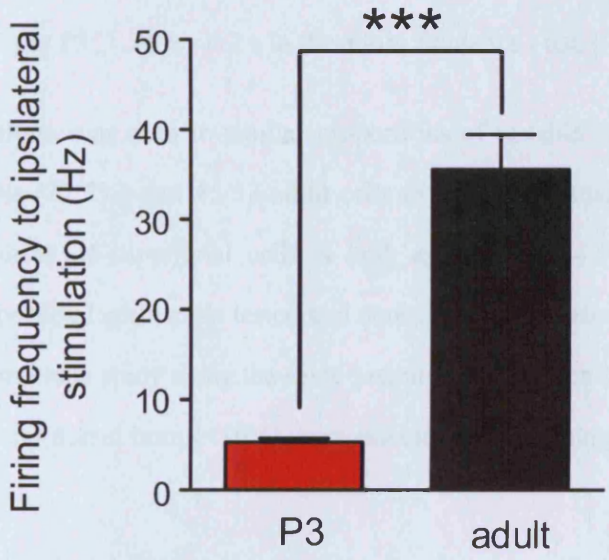


Figure 4.5 Baseline firing frequencies at the two ages
Baseline firing frequency to ipsilateral stimulation is significantly greater in adult cells than in P3 cells ($p < 0.0001$)

4.3.2 Contralateral inhibition is present at both ages

4.3.2.1 Contralateral pinch

Inhibition of evoked firing could be provoked by pinch of the contralateral hindpaw in both adult and P3 rats. This phenomenon has been observed previously in adult rats (Cadden et al, 1983; Fitzgerald, 1982; McGaraughty and Henry, 1997) but only once before in neonatal rat pups (Fitzgerald, 1985). Figures 4.6 and 4.7 illustrate the effect of contralateral pinch in example cells at both ages. As can be seen in these traces, the duration and intensity of the inhibition varied considerably on a cell by cell basis. The mean duration of inhibition was longer at P3, possibly due to the lower baseline firing rates against which the inhibition was tested (3.15 +/- 0.4 s at P3; 1.36 +/- 0.2 s in the adult; Student's t test $p < 0.0001$).

Contralateral inhibition was seen in similar proportions of testable dorsal horn cells at both ages: 24/33 P3 cells (72.7%) and 45/52 adult cells (86.5%). Interestingly, there was a high incidence of inhibition of superficial cells at both ages: 6/11 (54.5%) superficial P3 cells tested and all 5 superficial adult cells tested had contralateral inhibition (see figure 4.4). This is in contrast to a previous study using the same preparation in which only a low percentage of cells in the superficial dorsal horn (<10%) were inhibited by contralateral noxious stimulation (Fitzgerald, 1983).

The large difference between the two ages in the baseline frequency of firing evoked by the ipsilateral clip (see section 4.3.1) means that no direct comparison of the strength of inhibition can be made. When studying the region of maximal inhibition for each cell, the inhibition measured relative to the baseline appears significantly greater at P3 than in the adult (P3: 88.7 ± 2.1%; adult: 73.1 ± 2.8%; Student's t test $p < 0.001$; figure 4.8a). However, this is partly due to the much lower rate of baseline firing at P3 than in the adult (see section 4.3.1); when calculated in absolute terms, a greater number of spikes were inhibited in the adult than at P3 (P3: 15.5 ± 3.0 spikes; adult: 50.3 ± 10.6 spikes; Mann Whitney $p < 0.0001$; figure 4.8b).

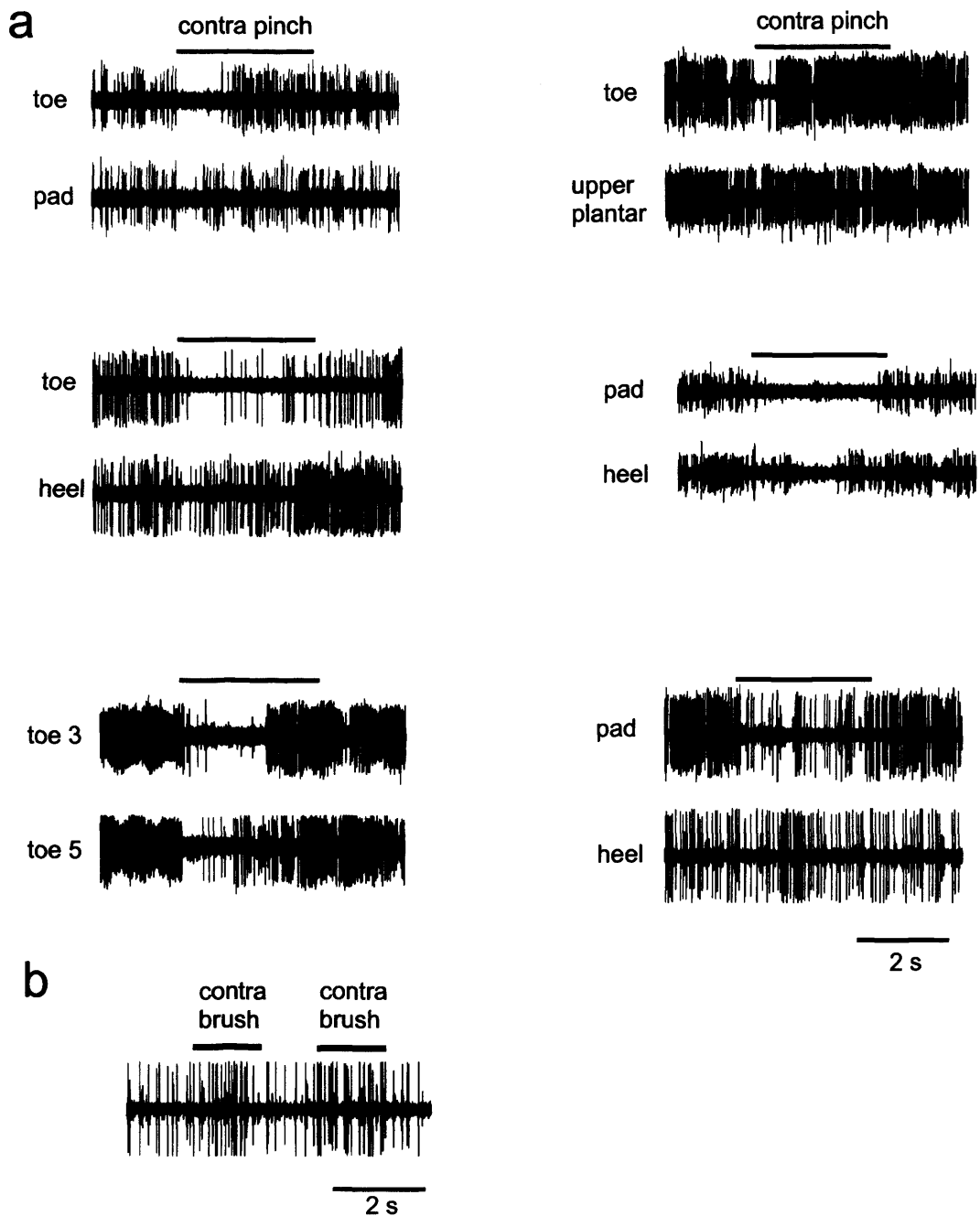


Figure 4.6 Sample traces showing contralateral inhibition in the adult

(a) Sample traces showing clear inhibition of firing to contralateral pinch in 6 adult cells. Two traces are presented for each cell to illustrate that pinching different contralateral plantar regions often produced different amounts of inhibition at this age. (b) Sample trace from one adult cell which showed excitation to contralateral innocuous brush.

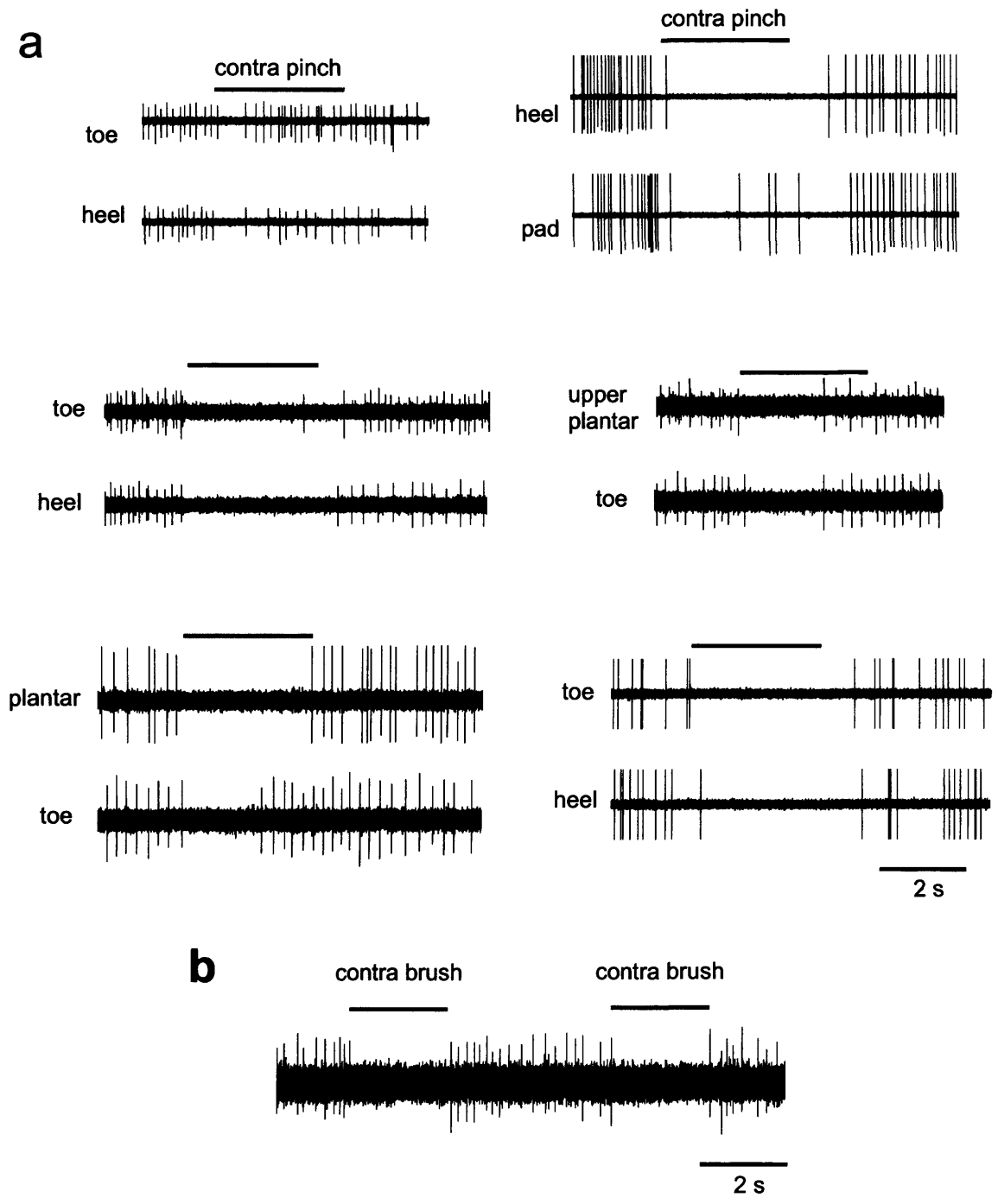


Figure 4.7 Sample traces showing contralateral inhibition at postnatal day (P) 3
(a) Sample traces showing clear inhibition of firing to contralateral pinch in 6 P3 cells. Two traces are presented for each cell to illustrate that pinching different contralateral plantar regions often but not always produced similar amounts of inhibition at this age. (b) Sample trace from one P3 cell which showed inhibition to contralateral innocuous brush.

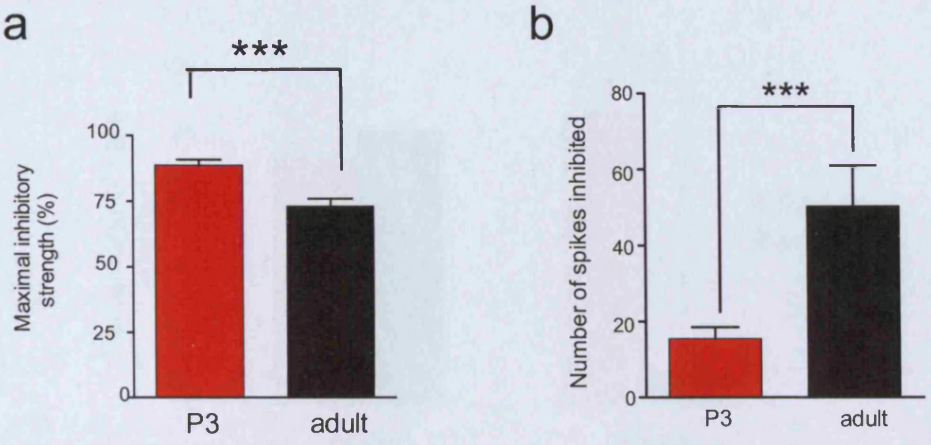


Figure 4.8 Comparisons of inhibitory strength
(a) When inhibition is measured relative to the baseline firing rate, the inhibition is significantly stronger at P3 than in the adult. (b) However, a significantly larger number of spikes were inhibited in the adult than at P3.

4.3.2.2 Contralateral brush

The effect of gentle brush of the contralateral hind paw was assessed in a subset of cells at both ages. A striking developmental difference was observed: this low-threshold, innocuous stimulus inhibited the spike train frequently at P3 (12/14 cells; 85.7%) but only rarely in the adult (2/20 cells; 10%). A X^2 test showed that this was highly statistically significant ($p < 0.0001$; see figure 4.9).

4.3.2.3 Rebound excitation

Following the cessation of inhibition, the firing rate was frequently higher than the baseline rate for a short while, before returning to baseline values (see figures 4.6 and 4.7 for sample traces). This was observed in 9/24 P3 cells (37.5%) and 22/45 adult cells (48.8%).

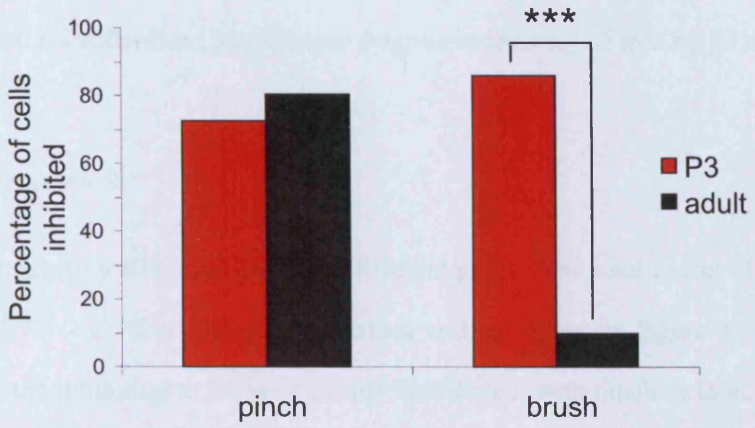


Figure 4.9 Modality specificity of contralateral inhibition in the adult but not at P3
The percentage of cells inhibited by contralateral pinch was similar at both ages, but contralateral brush inhibited a much higher percentage of cells at postnatal day (P) 3 than in the adult.

4.3.3 Inhibitory receptive fields

The limits of the excitatory and inhibitory receptive fields (RFs) were mapped on the ipsilateral and contralateral hind paws in the normal way. A careful analysis of the relative strength of inhibition in the different regions of the inhibitory RF was then conducted for quantitative spatial analysis of the inhibitory RF. This was possible in 21/45 cells in the adult and 17/24 cells at P3 (figures 4.10 and 4.11).

4.3.3.1 Adult inhibitory RFs

Adult inhibitory RFs had a large total extent, covering 87.8 +/- 4.8% of the plantar surface. However, the inhibition was not evenly distributed across this region but was quite spatially restricted: the strongest inhibition (0.81-1.00 bin) covered only 27.7 ± 5.4% of the plantar surface (see figure 4.12). As figure 4.10 shows, the contralateral inhibitory RF was often close to being a mirror image of the ipsilateral excitatory RF, confirming results in the literature

(Fitzgerald, 1982). The mean distance between the excitatory and inhibitory RF centres was 6.3 +/- 1.4 mm on a standardised plantar paw diagram measuring 35 mm by 13 mm.

4.3.3.2 P3 inhibitory RFs

The pattern of inhibitory RFs was strikingly different at P3. The total extent of the inhibitory RFs covered 96.9 +/- 2.3% of the plantar surface and, as shown in figure 4.11, there was a strong trend for the inhibition to be more evenly distributed, with pinch to large regions of the plantar surface tending to inhibit firing equally strongly: the strongest inhibition (0.81-1.00 bin) covered 69.1 ± 6.7% of the plantar surface (see figure 4.12). It is important to note that the bins for the inhibitory RF were calculated on a cell by cell basis specifically to investigate the degree of RF tuning; the larger regions of "strongest" inhibition do not necessarily mean that there was more inhibition at P3, just that the area of maximal inhibition was less spatially restricted. The excitatory and inhibitory RF centres were significantly less well aligned at P3 than in the adult: the mean distance between the excitatory and inhibitory RF centres was 13.2 +/- 1.6 mm on the standardised plantar paw diagram, more than twice the distance measured in the adult (Student's t test, $p < 0.001$; see figure 4.13).

4.3.3.3 Quantitative spatial analysis of RF tuning at P3 and adult

The spatial tuning of inhibitory RFs was measured for each cell by plotting the strength of normalised inhibition against cumulative plantar coverage and measuring the area under the resulting curve, with 1.00 representing equal inhibition across the whole plantar area. Figure 4.14a shows sample AUC plots for a cell at each age. The qualitative change in spatial tuning from P3 to adult discussed above was found to be strongly statistically significant when analysed in this way: mean AUCs were 0.90 ± 0.04 at P3 and 0.67 ± 0.04 in the adult (Mann Whitney $p < 0.001$; figure 4.14b).

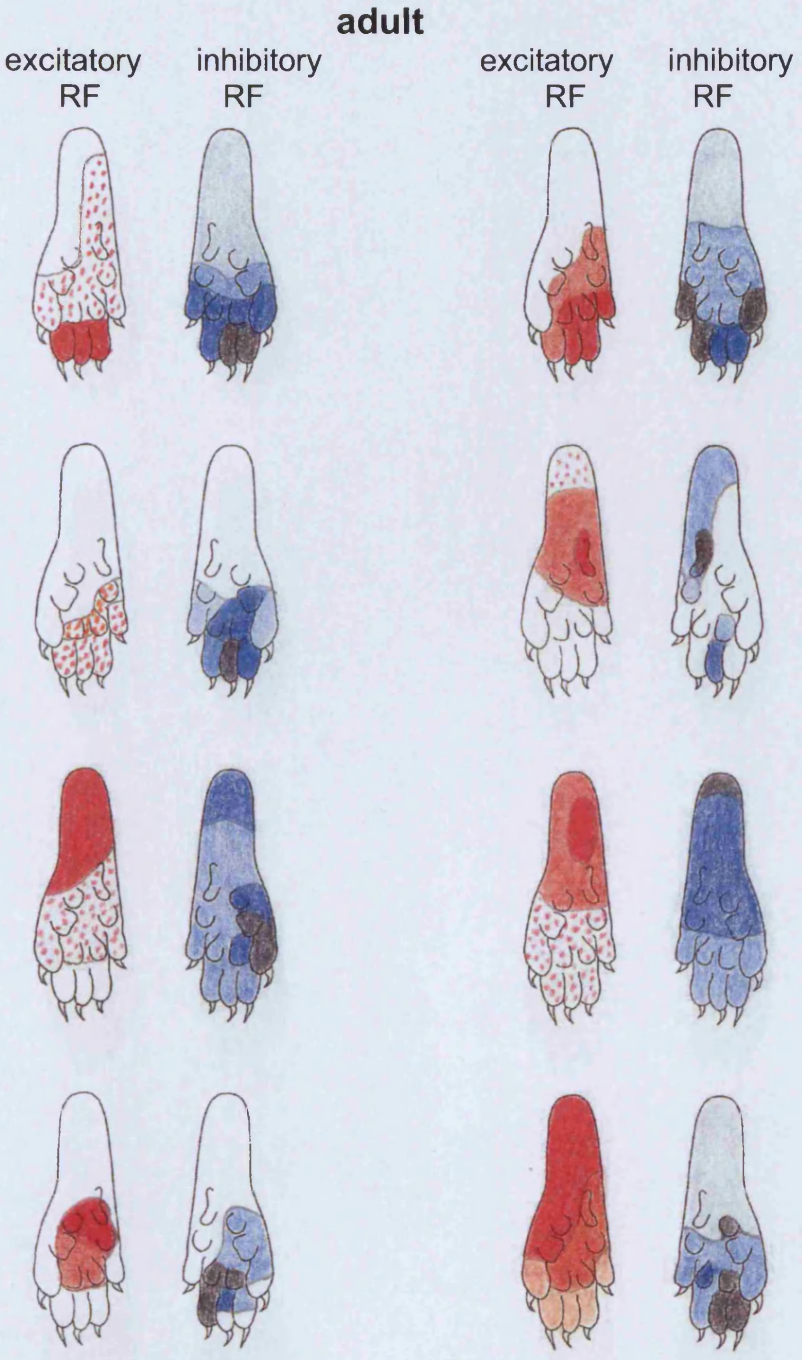


Figure 4.10 Ipsilateral excitatory and contralateral inhibitory receptive fields in the adult

Receptive fields (RFs) for all spatially mapped adult cells (n = 21; continued overleaf). Excitatory RFs (left, red and orange) were mapped qualitatively, with red denoting the region of greatest response and orange the regions of lesser response. Dotted regions responded only to pinch. Inhibitory RFs (right, shades of blue and black) were mapped quantitatively relative to the region of maximal inhibition (see text for more details, and key overleaf).

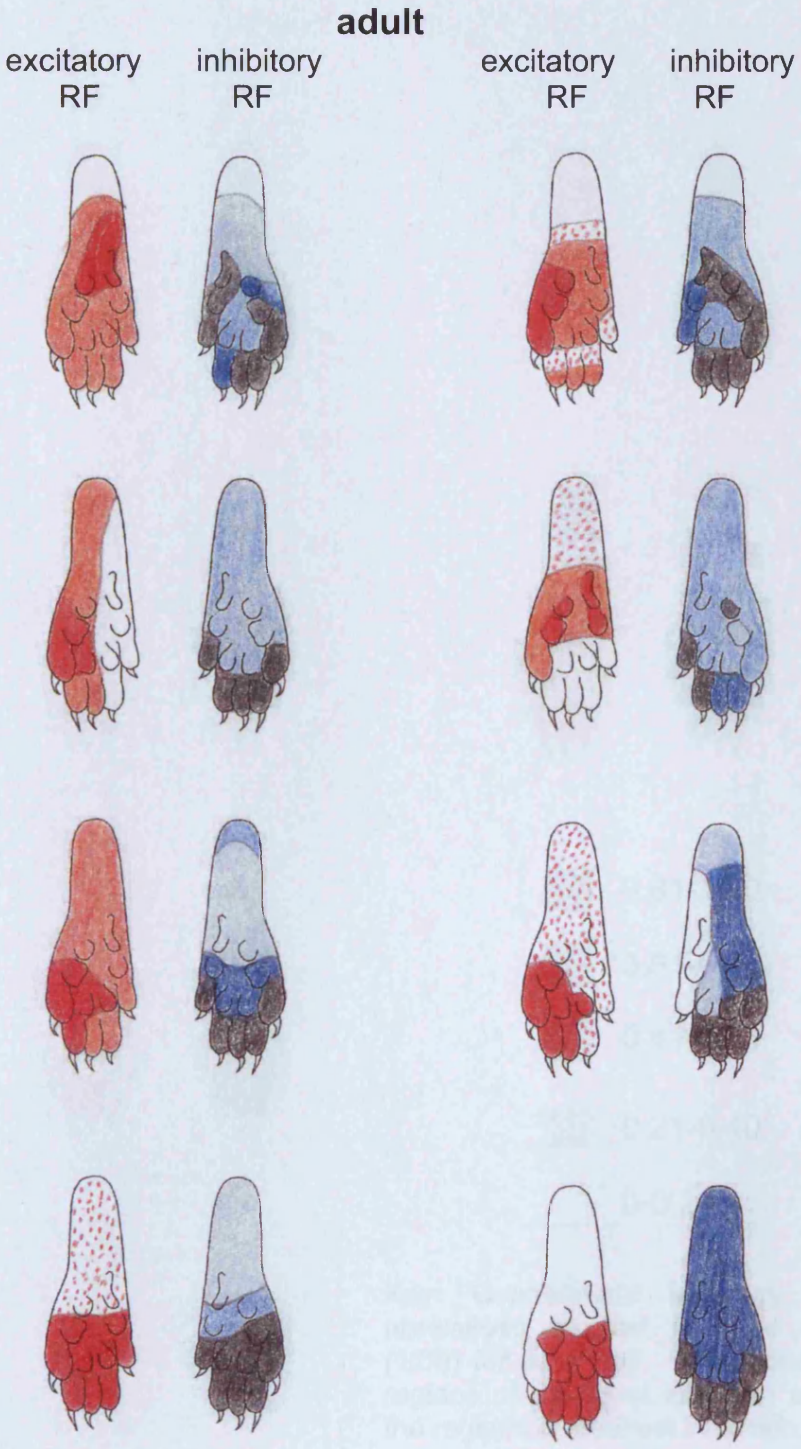
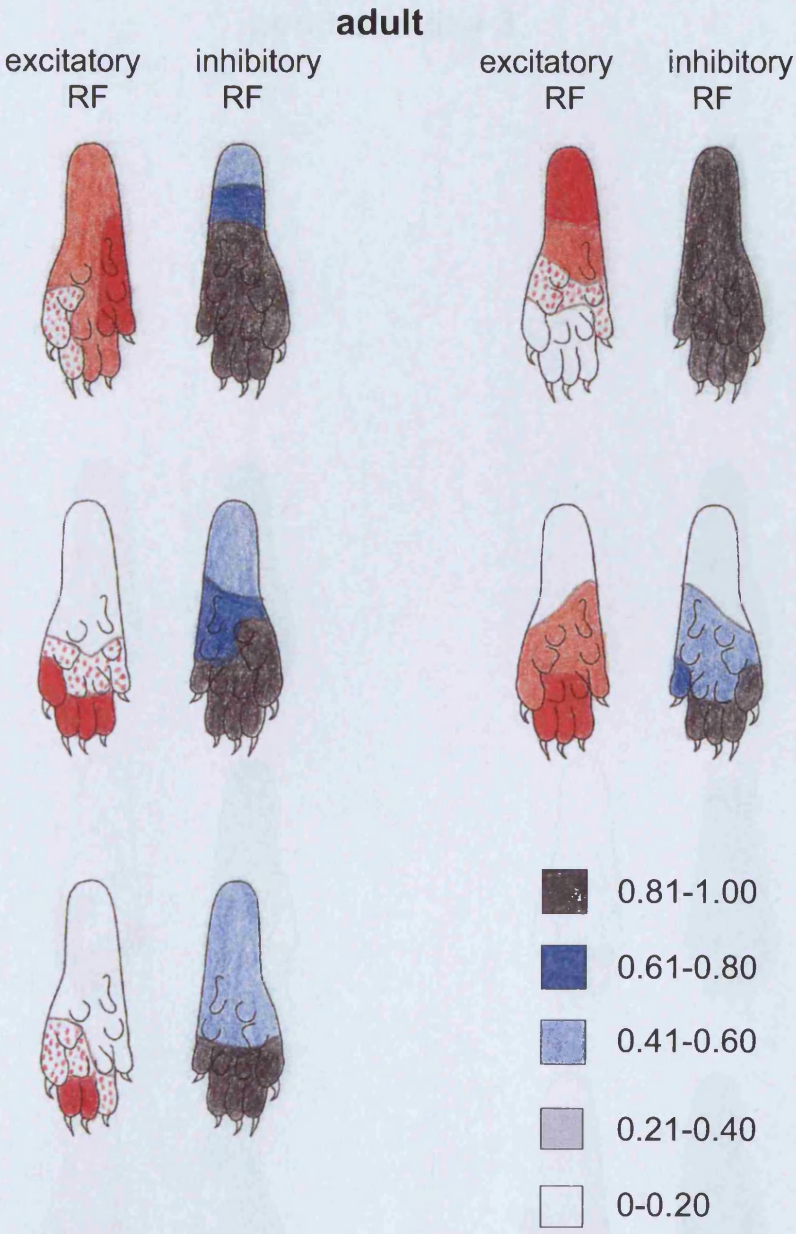


Figure 4.10... continued Adult excitatory and inhibitory receptive fields



Key: Contralateral inhibitory strength normalised to the maximal inhibition (1.00) for each cell. Black denotes the regions of strongest inhibition and white the regions of weakest inhibition.

Figure 4.10... continued Adult excitatory and inhibitory receptive fields

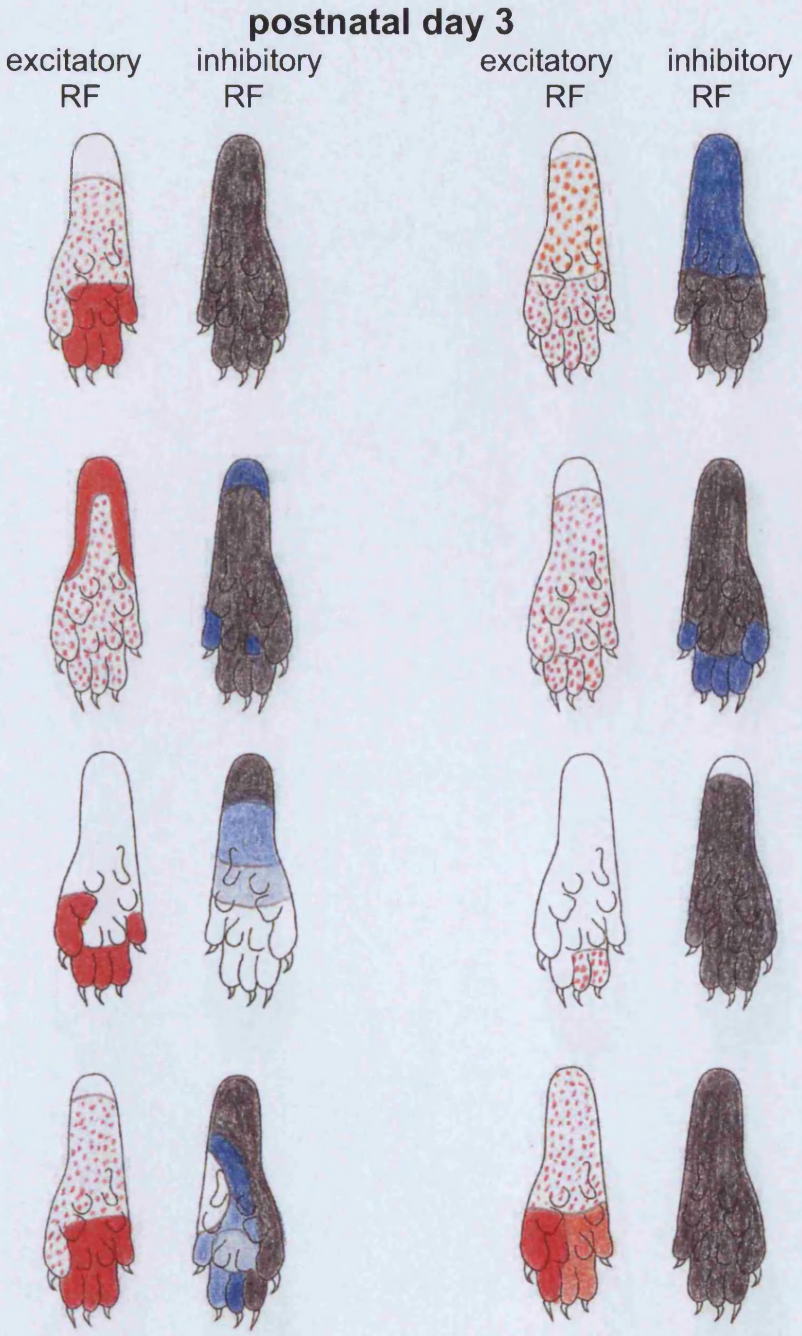


Figure 4.11 Ipsilateral excitatory and contralateral inhibitory receptive fields at postnatal day (P) 3

Receptive fields (RFs) for all spatially mapped P3 cells (n = 17; continued overleaf). Excitatory RFs (left, red and orange) were mapped qualitatively, with red denoting the region of greatest response and orange the regions of lesser response. Dotted regions responded only to pinch. Inhibitory RFs (right, shades of blue and black) were mapped quantitatively relative to the region of maximal inhibition (see text for more details, and key overleaf).

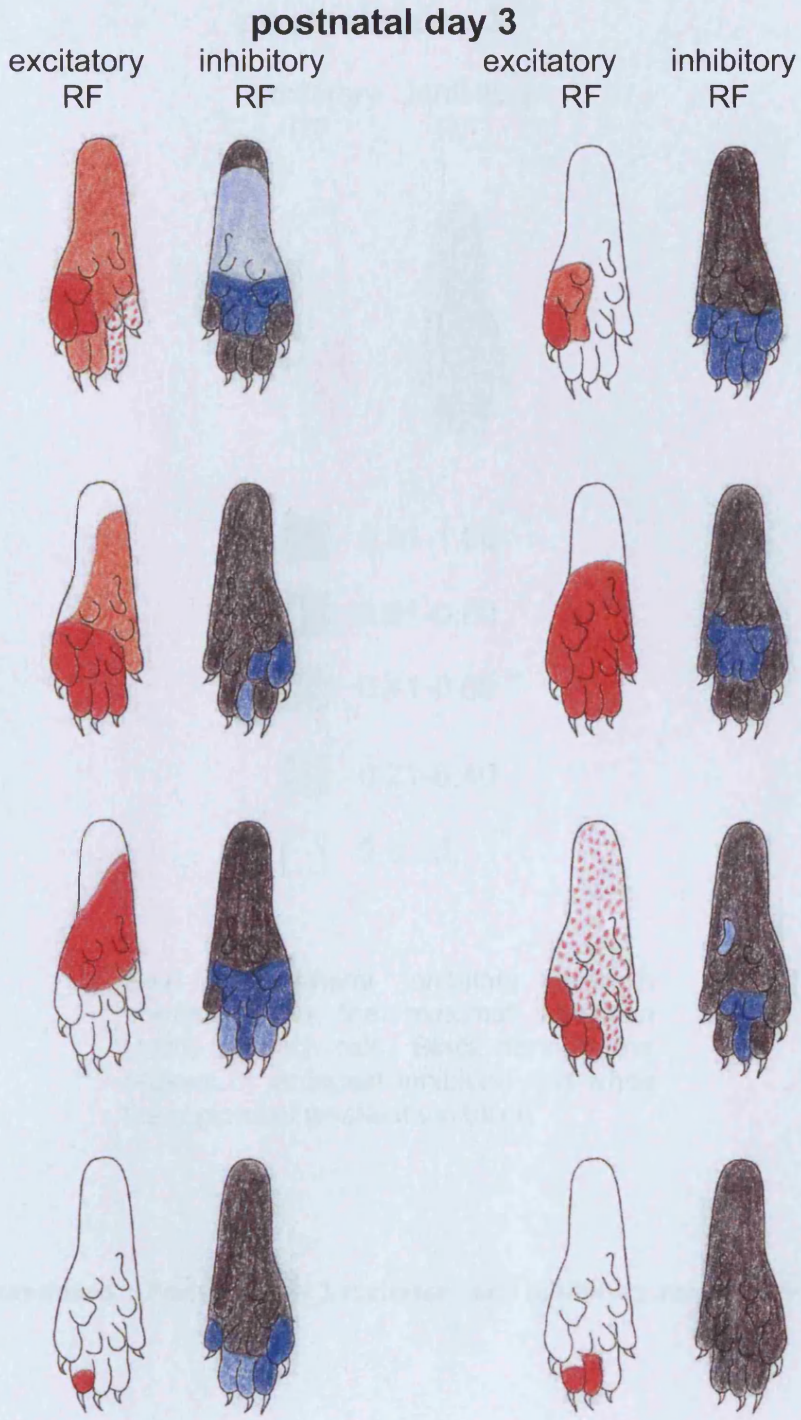
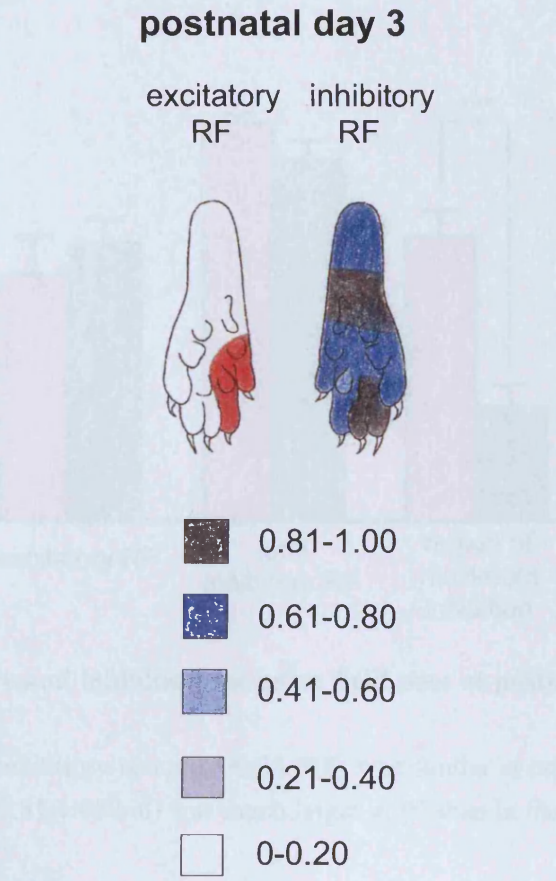


Figure 4.11... continued Postnatal day 3 excitatory and inhibitory receptive fields



Key: Contralateral inhibitory strength normalised to the maximal inhibition (1.00) for each cell. Black denotes the regions of strongest inhibition and white the regions of weakest inhibition.

Figure 4.11... continued Postnatal day 3 excitatory and inhibitory receptive fields

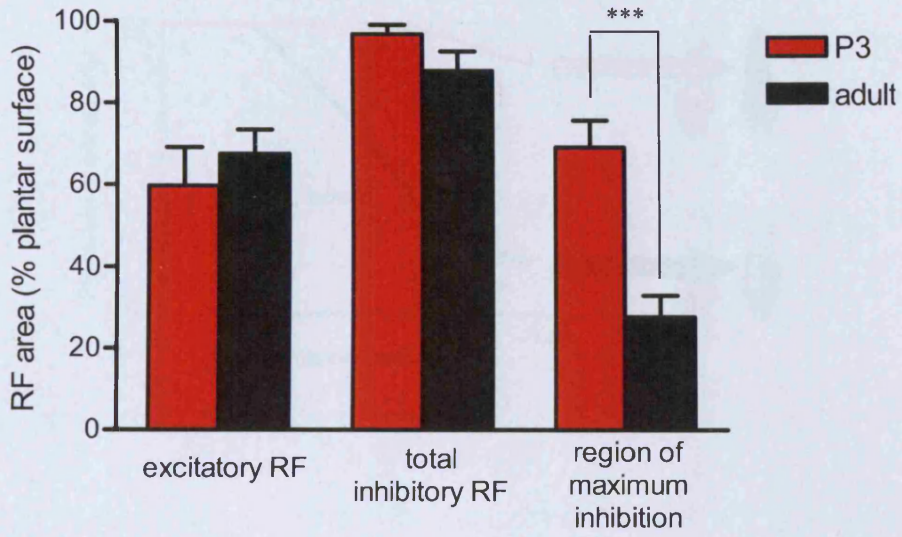


Figure 4.12 Excitatory and inhibitory receptive field sizes at postnatal day (P) 3 and in the adult.

The total extent of the inhibitory receptive field (RF) was similar at both ages, but the region of maximal inhibition (0.81-1.00 bin) was much larger at P3 than in the adult (Mann Whitney $p < 0.0001$).

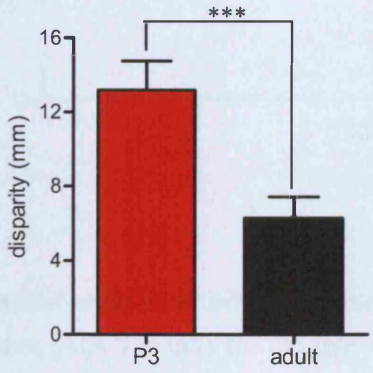


Figure 4.13 Excitatory and inhibitory receptive fields are poorly aligned at postnatal day (P) 3

The disparity between the centre of the regions of maximal excitation in the excitatory receptive field (RF) and maximal inhibition in the inhibitory RF was greater at P3 than in the adult (Student's t test $p < 0.001$).

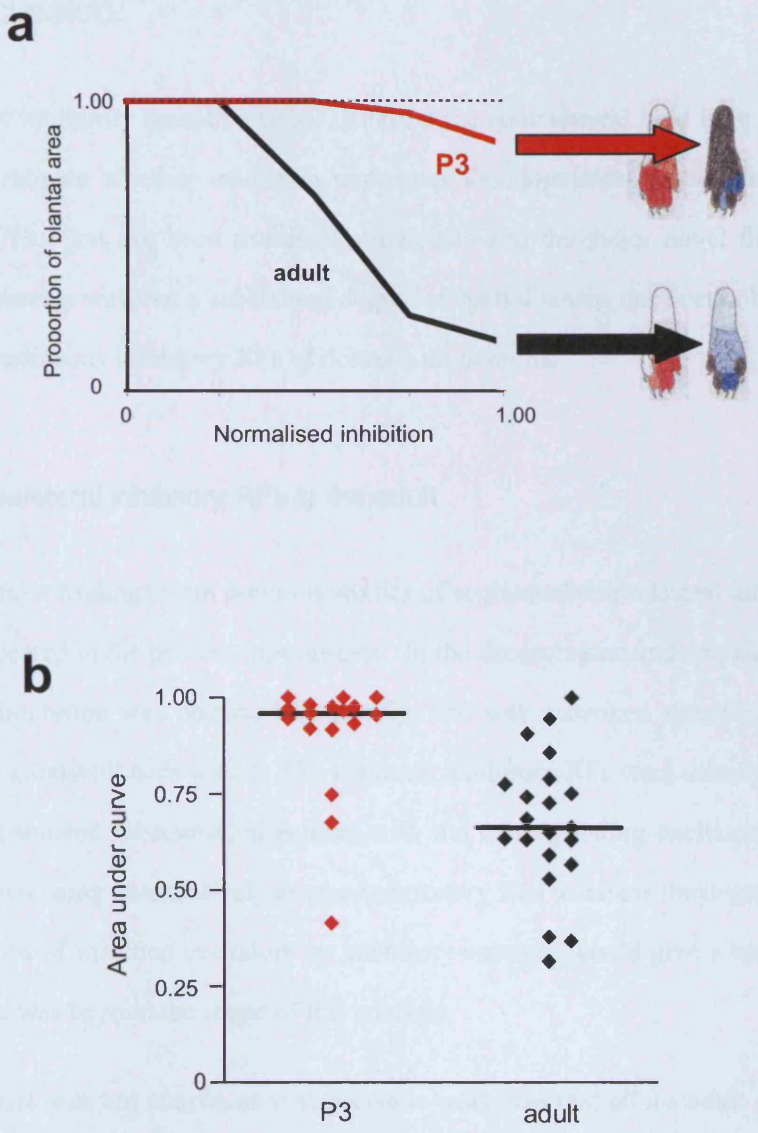


Figure 4.14 Quantitative spatial analysis of inhibitory receptive field tuning
(a) Sample area under the curve (AUC) graphs for the inhibitory RFs of a representative P3 and adult cell. (b) Total AUC for all inhibitory RFs mapped. Units are arbitrary, with 1.00 representing completely even inhibition across the whole plantar area. Horizontal lines represent the median. The difference between the two ages was highly significant (Mann Whitney $p<0.001$).

4.4 Discussion

In this chapter inhibitory receptive fields (RFs) on the contralateral hind limb were used as a model to investigate whether inhibition undergoes developmental spatial tuning in the rat dorsal horn. This has not been examined previously and the major novel finding from the present experiments was that a substantial degree of spatial tuning does occur between P3 and adult for the cutaneous inhibitory RFs of dorsal horn neurons.

4.4.1 Contralateral inhibitory RFs in the adult

Most of the major findings from previous studies of segmental contralateral inhibition in adult rats were replicated in the present experiments. In the decerebrated and spinalised preparation, contralateral inhibition was observed frequently, and was provoked specifically by noxious stimulation in almost all cells tested. The maximal inhibitory RFs were usually quite spatially restricted and showed substantial alignment with the corresponding excitatory RF. A more detailed analysis using quantitatively mapped excitatory RFs to assess the degree of separation between regions of matched excitatory or inhibitory strength would give a better measure of symmetry, but was beyond the scope of this analysis.

One result which was not consistent with previous work was that all the adult superficial cells tested in the present experiment showed contralateral inhibition. This contrasts with previous work in the same preparation which showed that <10% of cells in substantia gelatinosa were inhibited by contralateral stimulation (Fitzgerald, 1983). This discrepancy may be due to the low numbers of adult superficial cells tested in the present work (n = 5) compared with the earlier study (n = 43).

4.4.2 Contralateral inhibitory RFs at P3

Cutaneous inhibitory RFs have not previously been studied in detail in the neonate. It was possible that contralateral inhibition might not be functional at this early stage; however, the results clearly demonstrate that it is present in a similar proportion of cells at P3 as in the adult. There is therefore no simple deficit in this form of inhibition, which is consistent with the results from chapter 2 showing that GABAergic inhibition is functional in the early postnatal dorsal horn.

4.4.2.1 Comparison of the strength of contralateral inhibition at each age

The strength of contralateral inhibition at P3 appeared similar to that in the adult, but an accurate comparison of inhibitory efficacy was confounded by the large differences in baseline firing rate, against which the inhibition was measured. Cells at each age tended to fire within particular frequency ranges: 4 - 10 Hz for P3 cells and 20 - 50 Hz for adult cells. Ideally, the adult cells could have been stimulated just sufficiently to produce firing rates of <10 Hz so that the strength of inhibition at the two ages could be compared; in the experimental preparation used, however, stimulation of adult cells tended to produce either transient responses only or sustained firing at >10 Hz, so this was not possible. The much lower firing frequencies seen at P3 probably reflect the lower efficacy of excitatory transmission in the dorsal horn at early postnatal ages. Spontaneous EPSCs in the superficial dorsal horn are upregulated during the first ten postnatal days (Baccei et al. 2003), and C-fibre evoked activity is also weak or nonexistent until approximately P10 (Baccei et al, 2003; Jennings and Fitzgerald, 1998). That contralateral pinch at P3 is able to clearly inhibit the low-frequency sustained firing at this age indicates that the inhibition is not weak but is well balanced with the excitation in the dorsal horn at this age.

4.4.2.2 Postnatal development of modality specificity

One striking novel result from the present work was that contralateral innocuous brush often clearly inhibited the train of firing at P3, but only rarely in the adult. Care was taken to ensure that the brushing was very gentle and innocuous, so the results are not because the stimulus was innocuous in the adult but noxious at P3.

The lack of modality specificity of contralateral inhibition at P3 could be related to the innervation of the superficial laminae by Aβ fibres in the early postnatal period. This has been demonstrated both anatomically (Torsney et al. 2000; Beggs et al. 2002) and physiologically. Low-threshold stimulation produces Fos activation in superficial laminae at P3 but not at P21 (Jennings and Fitzgerald, 1996) and patch-clamp recordings from lamina II neurons showed a much greater incidence of monosynaptic Aβ-evoked inputs in spinal cord slices from P21 rats than from ~P60 rats (Nakatsuka et al, 2000). In the adult, the superficial laminae are innervated exclusively by small diameter fibres transmitting nociceptive information. Pathways which are specific for noxious stimulation in mature animals may therefore be activated by innocuous stimulation in immature animals, until the withdrawal of Aβ fibre terminals to deeper laminae. This could clearly influence contralateral inhibitory processing, particularly if the superficial laminae serve as the source of a commissural pathway which underlies segmental inhibition, as has been suggested (Fitzgerald, 1983). The ability of innocuous stimuli to provoke contralateral inhibition at P3 may serve to compensate for the reduced C-fibre input, and subsequent reduced C-fibre activation of contralateral inhibition, at this age. Interactions between A and C fibres in the superficial dorsal horn during development have been proposed to underlie the postnatal tuning of nociceptive reflexes by low-threshold tactile stimuli (Waldenstrom et al. 2003; Fitzgerald 2005), and the same processes may bring about the modality tuning of inhibitory receptive fields.

A degree of modality specificity is already known to be an important aspect of inhibitory dorsal horn processing: for example, the gating of noxious signals by ipsilateral low-threshold stimulation has been known for many years and has informed influential models of spinal cord

circuitry (Hillman and Wall, 1969; Melzack and Wall, 1965). It would be interesting to examine whether ipsilateral inhibition in the neonate is predominantly mediated by innocuous stimulation as in the adult, as a lack of modality specificity could indicate important differences in the processing and projection to higher centres of nociceptive information.

4.4.3 Postnatal development of inhibitory spatial tuning

The inhibitory RFs of P3 cells showed a marked lack of spatial tuning, with pinch to most areas of the contralateral plantar surface inhibiting firing equally well. This was in contrast to the inhibitory RFs of adult cells, which in most cases had a relatively small plantar region which produced the strongest inhibition. This finding is similar to results from *Xenopus* optic tectum, which showed a gradual refinement and matching of inhibitory and excitatory RFs over development (Tao and Poo, 2005), but there are also some differences. Although large, the immature inhibitory visual RFs of cells in the optic tectum still had quite a well-defined centre, which was misaligned with the excitatory RF centre. The inhibitory RFs of immature dorsal horn cells, in comparison, tended to be diffuse with no particular centre, and in general were 'misaligned' with the excitatory RFs due to their large, diffuse nature rather than because the RF was localised to a different plantar region.

4.4.3.1 Potential mechanisms for postnatal tuning of inhibitory RFs

Disrupting GABAergic signalling by either enhancing or reducing it delayed the developmental matching of RFs in the optic tectum, indicating that normal inhibitory neurotransmission is necessary for the refinement to occur in that system (Tao and Poo, 2005). This has also been demonstrated in the ocular dominance columns of the developing mammalian visual system, where the administration of benzodiazepines (which enhance efficacy at the $GABA_AR$) results in an acceleration of postnatal plasticity (Fagiolini and Hensch 2000). It would be interesting to see whether normal GABAergic / glycinergic activity is necessary for the developmental spatial tuning in the dorsal horn, but chronic

enhancement or reduction of GABAergic activity would be difficult to induce in the postnatal dorsal horn. GAD65 knockout mice, which have a deficit in GABAergic signalling but which remain viable, could prove useful for an investigation of this nature. In the auditory midbrain, activity-dependent changes in inhibitory synaptic strength are thought to play a role in the postnatal refinement of inhibitory circuits. Both high and low frequency stimulation can lead to long-term depression of inhibitory currents, with the former being dependent on functional $GABA_BRs$ (Kandler and Gillespie, 2005). $GABA_BRs$ are known to be present and functional in the early postnatal superficial dorsal horn (Baccei and Fitzgerald, 2004) and may be important for the maturation of inhibitory RFs in the spinal cord. In addition, they may prove to be particularly relevant for the tuning of nociceptive reflexes during development, which is known to be an activity-dependent process (Waldenstrom et al., 2003).

A second possibility is that the increased inhibitory tuning could be related to the emergence of glycinergic transmission during the second postnatal week. A developmental change in neurotransmitter phenotype from GABAergic to glycinergic also occurs in the MNTB – LSO pathway in the auditory system (Kotak et al., 1998; Nabekura et al., 2004), although the function of this switch is not yet known. GABAergic signalling is likely to play a role in contralateral inhibition because the phenomenom is clearly present at P3 when there are few glycinergic currents, in the superficial dorsal horn at least (Baccei and Fitzgerald, 2004), but the tuning may rely on the emergence of functional glycinergic synapses. This could be investigated by applying strychnine to the adult cord and measuring any change in the degree of tuning of the inhibitory RF. Should the adult inhibitory RFs with strychnine resemble those seen at P3, this would be consistent with a role for glycine in tuning the inhibitory RFs.

The postnatal reduction in excitatory RF size has been shown to be NMDAR dependent: chronic application of the NMDAR antagonist MK-801 to the spinal cord from birth to ~P55 prevented the reduction in excitatory RF size which normally occurs over the first three postnatal weeks (Beggs et al, 2002). It is possible to envisage a simple circuit whereby cells in opposite sides of the dorsal horn with equivalent ipsilateral excitatory RFs are linked by

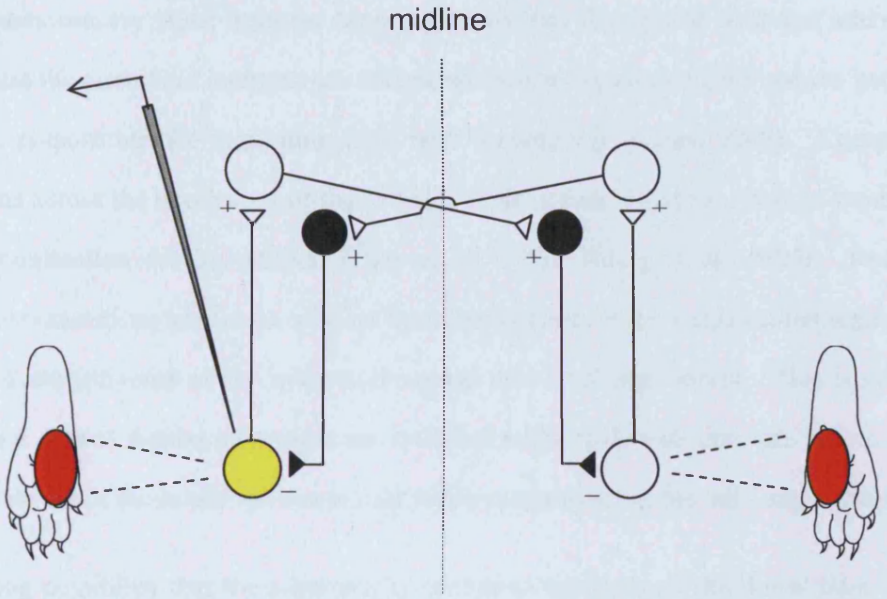


Figure 4.15 Diagram to illustrate one possible simple circuit that may underlie contralateral inhibition.

In this circuit, pairs of cells with symmetrical excitatory receptive fields are linked by mutual inhibition, so that the excitatory RF of one cell becomes the inhibitory RF of the paired cell. The recorded cell is shown here in yellow, white represents excitatory interneurons (+), some of which cross the midline of the spinal cord, and black represents inhibitory interneurons (-).

reciprocal inhibition (see figure 4.15). In such a situation, the developmental tuning of contralateral inhibitory RFs would rely directly on the same processes which underlie the reduction in excitatory RF size. Even if such specific links do not exist, the circuits controlling the inhibitory RFs are likely to contain excitatory interneurons as well as inhibitory interneurons, and so the developmental tuning observed in the present study may rely upon NMDAR activity.

4.4.4 Speculations on the functional role of contralateral inhibition

The inhibition of firing produced by contralateral pinch is profound and robust, yet its function remains largely unknown. The research on this segmental inhibition has been conducted in the lumbar region of the spinal cord. The dorsal horn in these segments receives

and processes sensory input from the hind limbs, whereas the ventral horn and intermediate zone contain the circuits of interneurons and motoneurons known as central pattern generators, which are responsible for organising hind limb locomotion (Kiehn, 2006). Commissural connections across the two halves of the spinal cord are necessary at this level to produce left – right coordination for locomotion (Butt et al, 2002; Zhong et al, 2006). Reciprocal glycinergic connections across the midline from interneurons in the ventromedial region of the spinal cord are activated as the ipsilateral ventral root discharge occurs. This ensures that contralateral central pattern generators are inhibited while ipsilateral ones are active, and the reciprocal nature of the inhibition is a crucial factor in maintaining the left – right alternation.

It is a strong possibility that the cutaneous contralateral inhibition in the dorsal horn reported in this chapter is linked to these locomotor patterns. During locomotion, cutaneous input will alternate from the left to the right hind paw as each makes contact with the surface and is then removed. Inhibition of the contralateral dorsal horn during the time that the ipsilateral paw is in contact with the walking surface could serve to enhance the ipsilateral signal, and this information could influence the ventral horn processing as well as rostral projections. One argument against this hypothesis is that the cutaneous input from walking is rarely nociceptive, so the inhibition would not normally be initiated in the adult. The hypothesis that the function of segmental contralateral inhibition in dorsal horn neurons is primarily related to locomotion could be tested by recording from cells in other segments of the spinal cord that receive afferents from skin regions which do not play such a major role in locomotion, for example the trunk. If such cells do not possess segmental contralateral inhibitory RFs, then the contralateral inhibition found in lumbar regions would appear to be specialised for the hind limbs and probably locomotion. However, if such cells did possess similar contralateral inhibitory RFs then the inhibition would appear to have a more general function, such as shaping cutaneous reflexes or enhancing sensory processing.

Cutaneous reflex activity can be inhibited by noxious stimulation of areas outside the excitatory RF, including the contralateral hind paw. This inhibition is thought to be important

for the prevention of inappropriate withdrawal reflexes and, in the case of the inhibition by contralateral stimulation, may be involved in the crossed extensor reflex pathways which support the body during withdrawal reflexes (Weng and Schouenborg, 1996). The excitatory cutaneous reflex RFs in immature animals are disorganised and neonatal reflex behaviour is imprecise and poorly directed (Andrews et al, 2002; Holmberg and Schouenborg, 1996; Waldenstrom et al, 2003). This may be partly due to immaturity in the inhibitory cutaneous reflex RFs, which are probably shaped to some degree by the inhibitory RFs of neurons in the dorsal horn. It would be interesting to study inhibitory cutaneous reflex RFs in neonatal rats, with one prediction being that the contralateral RFs would be also be disorganised compared with those in the adult.

In terms of sensory processing, the contralateral inhibition may serve to produce a more spatially distinct representation of a noxious stimulus. As illustrated in the model in figure 4.16, pinch to one hind paw will produce increased excitation in ipsilateral dorsal horn cells which have a receptive field covering the stimulated area. This is represented in the diagram by the red and yellow regions ipsilateral to the pinch. The same pinch will simultaneously decrease firing in a set of contralateral dorsal horn cells, represented in the diagram by the contralateral blue regions. Ongoing background activity in both ipsilateral and contralateral dorsal horn cells unaffected by the pinch is represented by paler yellow and the number 1. In the adult, the two patterns of increased and decreased activity are relatively symmetrical, and a hypothetical "comparator" could subtract one representation from the other to filter out background activity and produce a more defined representation of the pinch. In the neonate, however, the contralateral inhibition is less spatially tuned and so the same comparison would result in a more diffuse and excitable representation. A similar 'information enhancing' role has been proposed for the diffuse noxious inhibitory controls onto WDR cells (Willis and Coggeshall 1991; see section 4.1.1.2). It is important to note that the present model relies upon the excitatory and inhibitory RFs being symmetrical, which the present data support to some extent, but not entirely (see figure 4.10). In the neonate, the inhibition was less spatially tuned, and the same comparison would result in a more diffuse and excitable end

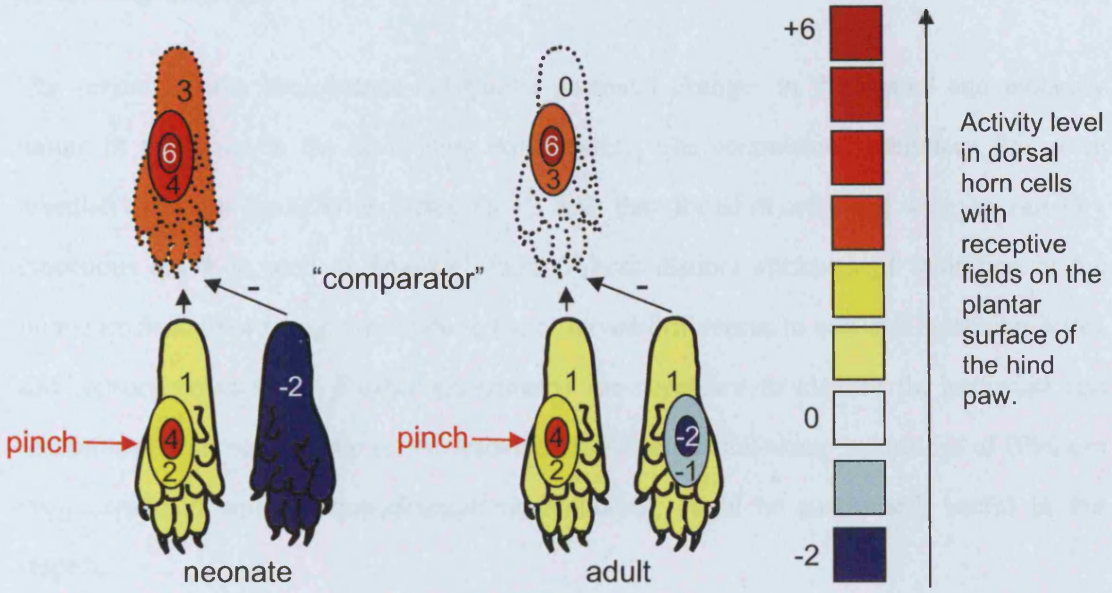


Figure 4.16 Model of the potential consequences of postnatal inhibitory spatial tuning
Pinch to one hind paw will produce increased excitation in ipsilateral dorsal horn cells which have a receptive field covering the stimulated area. This is represented in the diagram by the red and yellow regions ipsilateral to the pinch. The same pinch will simultaneously decrease firing in a set of contralateral dorsal horn cells, represented in the diagram by the contralateral blue regions. Ongoing background activity in both ipsilateral and contralateral dorsal horn cells unaffected by the pinch is represented by pale yellow and the number "1". In the adult, the two patterns of increased and decreased activity are relatively symmetrical, and a hypothetical "comparator" could subtract one representation from the other to filter out background activity and produce a more defined representation of the pinch (right). In the neonate (left), the contralateral inhibition is less spatially tuned and so the same comparison would result in a more diffuse and excitable representation.

representation. For clarity, the pinch in figure 4.16 is shown producing the same pattern of ipsilateral excitation at P3 as in the adult. Given the larger excitatory RFs at P3, and hence greater presumed overlap of RFs, a pinch would be expected to produce a larger initial excitatory representation at this age. However, the inhibitory RFs are commonly so diffuse at this age that the essence of the model still holds.

4.4.5 Conclusions

The present results demonstrate substantial postnatal changes in the spatial and modality tuning of inhibition in the developing dorsal horn. The contralateral inhibitory RFs were revealed to be less spatially restricted for P3 cells than for adult cells, and were activated by innocuous brush as well as noxious pinch. These distinct attributes of inhibition in the immature dorsal horn may contribute to the observed differences in neonatal cutaneous reflex and sensory processing. Further experiments are necessary to identify the pathways and neurotransmitters responsible for contralateral inhibition; patch-clamp recordings of IPSCs *in vivo*, combined with pharmacological manipulations, could be particularly useful in this respect.

Chapter 5

General discussion

5.1 Introduction

The neonatal nervous system is not merely a scaled-down version of the adult nervous system but is qualitatively different and undergoes a considerable degree of postnatal development. The work presented in this thesis demonstrates this for the inhibitory circuitry in the dorsal horn of the spinal cord. In the somotosensory and nociceptive system, reflexes and behaviour are subject to postnatal changes in threshold, duration and tuning (Fitzgerald et al. 1988; Andrews and Fitzgerald 1994; Holmberg and Schouenborg 1996; Andrews et al. 2002; Waldenstrom et al. 2003; Walker et al 2005). It was hypothesised that these developmental changes are due to maturation of the inhibitory circuitry in the dorsal horn. Cellular and molecular studies have previously shown that many changes occur in dorsal horn inhibition over the postnatal period including an upregulation in the frequency of inhibitory postsynaptic currents (IPSCs), an increasing role for GlyR-mediated IPSCs, changes in the kinetics of both GlyR and $GABA_AR$-mediated IPSCs, and an increasing capacity for chloride extrusion (Takahashi et al. 1992; Baccei and Fitzgerald 2004; Keller et al. 2004; Cordero-Erausquin et al. 2005). However, the functional significance of these changes in the context of the whole animal remained to be assessed. The aim of this thesis was therefore to study the maturation of dorsal horn inhibitory processing *in vivo* at different postnatal ages. The experiments presented here demonstrate that spinal GABAergic transmission is functional in rat pups by the third postnatal day, but that descending controls and network-level inhibitory circuitry in the dorsal horn remain immature at this age.

5.2 Summary of results

The maturation of segmental and descending inhibitory processing in the rat spinal cord was studied using *in vivo* extracellular electrophysiological recording of single dorsal horn cells. It has been suggested that a postnatal shift in the transmembrane anion gradient followed by a period of immaturity in chloride transport mechanisms may result in a deficit in the ability of

GABA to inhibit dorsal horn neurons over the first three postnatal weeks (Cordero-Erausquin et al. 2005). The experiments described in chapter 2 tested this for the first time *in vivo* by recording the properties of dorsal horn cells at postnatal day (P) 3 and P21 in the absence and presence of gabazine, a $GABA_AR$ antagonist. At both P3 and P21, gabazine caused a robust disinhibition, measured as an enlargement of RF areas and increased firing to threshold and suprathreshold cutaneous stimulation, indicating the presence of functional GABAergic inhibition at both ages. This finding does not negate the importance of the many developmental differences observed at the cellular and molecular levels; it is likely that these subtle differences do contribute to the control of inhibition over development. For example, the inhibition at P3 could be mediated less by membrane hyperpolarisation than by shunting, and this may be appropriate for the state of the system at this age, especially as the shunting is probably enhanced by slow IPSC decay rates in the neonate (Keller et al. 2004). However, the results from chapter 2 demonstrate that the excitability of neonatal nociceptive circuits is not due to an inability of GABA to inhibit dorsal horn neurons, but may instead reflect immaturity in the circuit and systems-level organisation of inhibition.

One obvious systems-level candidate for such a postnatal change is the supraspinal control of the dorsal horn, which is known to exert a strong tonic inhibitory influence on spinal reflexes and single dorsal horn cells in the adult (Sherrington and Sowton 1915; Wall 1967; Laird and Cervero 1990). Moreover, it has been known for some time that this descending inhibition is weak or absent in the rat during the first two to three postnatal weeks (Fitzgerald and Koltzenburg 1986; van Praag and Frenk 1991; Boucher et al. 1998). The experiments described in chapter 3 extended this with the novel finding that electrical stimulation of descending tracts (the dorsolateral funiculus or DLF) can provoke both inhibition and facilitation of evoked firing at P3, as well as sometimes being without effect. The effect of DLF stimulation on spontaneous firing of dorsal horn cells was also investigated for the first time and, surprisingly, was found to inhibit ongoing activity in both P3 and adult cells. Despite this latter finding, it is clear that substantial postnatal changes occur in descending

modulation of the spinal cord, which are likely to influence the behavioural and reflex responses to nociceptive stimuli at different ages.

The last set of experiments investigated the segmental organisation of inhibition in the dorsal horn by mapping the inhibitory receptive fields of dorsal horn cells in the spinalised P3 pup and adult. Contralateral inhibitory RFs were used as model to test whether inhibitory RFs undergo postnatal spatial tuning, and the results clearly showed that a substantial degree of both spatial and modality tuning occurs over the postnatal period. Importantly, however, there was no evidence that the strength of this contralateral inhibition was any weaker at P3 than in the adult, confirming that inhibitory mechanisms are efficacious in the neonatal dorsal horn. The contralateral inhibitory RFs were found to be less spatially restricted for P3 cells than for adult cells and were activated by innocuous brush as well as noxious pinch, which together suggest that the underlying circuits controlling the organisation of inhibition are not yet mature in the neonatal rat. This may, in combination with the immaturity in descending controls, contribute to the observed differences in neonatal cutaneous sensory processing, and may be particularly important for the tuning of reflexes and excitatory RFs.

5.2.1 Experimental considerations

In line with the majority of *in vivo* electrophysiological experiments, the experiments in this thesis were somewhat restricted by the lack of precise identification of recorded cell types. Although cells were classified according to their responses to peripheral stimulation, as defined by Menetrey et al. (1977), and by depth, it was not possible to identify their morphology or precise lamina location. The dorsal horn is characterised by significant heterogeneity in the neuronal population, even within a given lamina, and it would have been desirable in the experiments to be certain that identical cell types were sampled at all ages. However, morphology is not necessarily an indicator of cell function in the dorsal horn (Light et al 1979; Woolf and Fitzgerald 1983; Light and Kavookjian 1988; Rethelyi et al. 1989) and the restricted analyses in this thesis failed to find any correlation between the particular

parameters assessed and recording depth. The use of sufficient sample sizes at all ages should also minimise any sampling biases.

It would have been of interest to know whether sampled cells were projection neurons or interneurons and, if the latter, whether the activity of excitatory or inhibitory interneurons was being recorded. This could be particularly important for the study of supraspinal modulation as the result of stimulation of descending pathways ultimately depends on the nature of the cell upon which the descending modulation acts. It is possible to make *in vivo* recordings in the intact animal and subsequently label the recorded cell for later location and immunocytochemical analysis. This can be done either by using the *in vivo* patch-clamp technique with biocytin or another marker in the patch pipette, or by making sharp intracellular recordings again with a glass electrode containing, for example, Lucifer Yellow (Woolf and Fitzgerald 1983). A similar technique is termed juxtacellular labelling and uses a glass pipette filled with biocytin to make extracellular recordings. Upon completion of electrophysiological recording, the electrode is moved very close to the cell and pulses of positive current passed across the membrane, allowing the biocytin to pass into the cell (Pinault 1996). The use of *in vivo* patching and juxtacellular labelling could prove useful in trying to bridge some of the gaps in knowledge between properties of identified cells recorded in slice preparations and the cutaneous response properties of cells recorded *in vivo*; however, such experiments were beyond the scope of this thesis.

A second caveat common to most *in vivo* recordings in the dorsal horn is the use of general anaesthetic agents. These are necessary to perform the laminectomy, to obtain stable recordings, and to enable appropriate characterisation of the cutaneous properties of each cell. Although the mechanism of action of many general anaesthetics is still unknown, there is clearly an effect on neuronal firing properties. This is particularly pertinent for the experiments in this thesis as one locus of action is likely to be an enhancement of inhibitory processing (Franks 2006). However, the same dose of anaesthetic (urethane) was administered at both ages in the experiments in chapter 2, so the comparisons between the two

ages remain valid, and the experiments in chapters 3 and 4 were conducted in a decerebrated preparation in which anaesthesia is withdrawn, so any effects here should be minimal. In any event, results from one preparation, such as an anaesthetised *in vivo* preparation, should always be considered in the context of results from other types of preparation, such as behavioural and *in vitro* experiments.

5.3 Balance of inhibition and excitation

5.3.1 Interpretation of the current results

Although there is much evidence for an increased excitability of dorsal horn circuits in the neonate compared with the adult, there is also an increasing recognition of the necessity for balance between excitation and inhibition during development (Fitzgerald 2005). The experiments in this thesis provide indirect support for this at a number of levels. Firstly, the fact that GABA can effectively inhibit dorsal horn cells despite a low efficacy of inhibitory transmission, as demonstrated by low frequencies of spontaneous and miniature IPSCs over the early postnatal period (Baccei and Fitzgerald 2004), and immature chloride homeostasis (Baccei and Fitzgerald 2004; Cordero-Erausquin et al. 2005; E. Harrop and M. Fitzgerald, unpublished observations) may be a result of the balancing of inhibition with excitation, the efficacy of which is also low at birth (Jennings and Fitzgerald 1998; Baccei et al. 2003). Adult-like inhibition in the dorsal horn during the early postnatal period could result in excessive dampening of the limited excitation at this time; concurrent development of excitation and inhibition appears to allow appropriate functionality of the inhibitory system at all postnatal ages.

Similarly, the low rates of spontaneous activity observed in P3 pups *in vivo* can be fully inhibited by stimulation of descending pathways (chapter 3) and by contralateral pinch (chapter 4), which indicates that these inhibitory mechanisms can be as effective in silencing spontaneous activity in the neonate as they are in the adult. It is not clear whether either form

of inhibition would be as effective at P3 if the frequency of spontaneous activity was as high as observed in the adult, so it is not possible to draw firm conclusions. However, the evidence that descending inhibition of evoked activity is weak in the neonate indicates that this inhibitory system is somewhat matched to the levels of excitation in the cord during development. This suggestion is strengthened by the putative link between development of the C fibre input to the cord and maturation of descending inhibition (Cervero and Plenderleith 1985). C fibres can mediate a strong excitatory input to the dorsal horn, so it would be inappropriate for powerful descending controls to exist prior to maturation of this input.

The effectiveness of contralateral inhibition in the neonate was surprising, given that in the adult it is mediated solely by noxious cutaneous stimulation or by stimulation of the sciatic nerve at C fibre strengths (Fitzgerald 1982). It would be predicted from the deficit in C fibre input during the first two postnatal weeks that this form of inhibition would be weak in the neonate. However, the ability of contralateral innocuous stimuli to provoke inhibition at P3 may provide a compensatory mechanism, again allowing the system to be balanced at this age.

Lastly, the marked lack of spatial tuning in the contralateral inhibitory RFs at P3 (chapter 4) is reminiscent of the enlarged excitatory RFs observed in the neonate (Fitzgerald 1985; Fitzgerald and Jennings 1999; Torsney and Fitzgerald 2002; see also section 2.3.1.2). Such enhancement of inhibitory RFs in the neonate could be seen as a means to balance the excitation produced by the large, and therefore also more overlapping, excitatory RFs. It is possible that similar NMDAR-dependent mechanisms underlie the postnatal tuning of both types of RF (Beggs et al. 2002), although this has not yet been tested for inhibitory RFs.

5.3.2 Activity-dependent plasticity in developing sensory systems

The notion of balanced excitation and inhibition through development is supported by studies from several sensory systems. Many of these address the issue through the examination of critical periods. These are limited windows of time during development in which activity-dependent plasticity occurs such that the system can be permanently altered. Correct sensory tuning in many modalities relies upon the central nervous system receiving the appropriate patterns of activity during the critical period. For example, visual deprivation during the critical period for the visual system produces cortical changes which result in permanently poor acuity in the previously deprived eye (Wiesel and Hubel 1963). Visual deprivation alters the cortex both structurally and physiologically: there is a physical expansion of the ocular dominance columns responding to the open eye and a shrinking of those subserving the deprived eye (Antonini et al. 1999; Trachtenberg and Stryker 2001). Visual deprivation is also known to scale up the strength of excitatory synapses, presumably in an attempt to compensate for the reduced excitatory drive (Fox and Wong 2005; Desai et al. 2002). Similarly, inhibition in the cortex is reduced after visual deprivation, as measured with both electrophysiological and immunohistochemical techniques (Hendry and Jones 1986; Benevento et al. 1995; Morales et al. 2002; but c.f. Maffei et al. 2006). Analogous decreases in inhibition after sensory deprivation have been reported in both the somatosensory and the auditory systems. Continuous removal of whiskers from birth results in a decrease in the number of GABAergic synapses in the rat barrel cortex (Micheva and Beaulieu 1995), and bilateral hearing loss during the gerbil critical period produces a reduction in inhibitory synaptic strength in both the inferior colliculus and the auditory cortex (Vale et al. 2003; Vale and Sanes 2000; Kotak et al 2005) as well as an increase in excitatory synaptic strength (Vale et al. 2002). These results all indicate that a driving principle of the developing sensory nervous system is maintenance of a suitable balance of activity: sensory deprivation paradigms result in a dramatic reduction in excitatory input and subsequent attempts to rebalance the system through up and downregulation of excitatory and inhibitory activity respectively.

In the visual system at least, the onset of the critical period for ocular dominance plasticity is itself determined by GABAergic activity. GAD65 knockout mice are viable but with reduced GABA release and fail to enter the critical period (Hensch et al. 1998), and the timing of the critical period in naive mice can be accelerated by infusion of benzodiazepine agonists, which enhance GABA efficacy (Fagiolini and Hensch 2000). In addition to determining the timing of the critical period, inhibition in the cortex, and particularly lateral inhibition, is important for determining the ultimate spacing of the ocular dominance columns. Chronic infusion of benzodiazepines during the critical period results in wider columns in the adult and, conversely, infusion of inverse agonists at the $GABA_AR$ results in narrower columns (Hensch and Stryker 2004).

It would be of great interest to observe the effects of cutaneous sensory deprivation on the development of both inhibition and excitation in the dorsal horn, especially as a critical period for the activity-dependent tuning of nociceptive tail reflexes has been found to exist during the early postnatal period (Waldenstrom et al. 2003). Compensatory mechanisms are known to be present in the embryonic chick spinal cord: when spontaneous network activity is blocked by lidocaine in ovo, there is an increase in the strength of glutamatergic and GABAergic synapses, both of which are excitatory at this stage of development (Gonzalez-Islas and Wenner 2006). It is therefore plausible that mechanisms for activity-dependent developmental plasticity of synapses may be present in the neonatal mammalian dorsal horn. The tuning of nociceptive reflexes during the critical postnatal period is known to be driven, paradoxically, by low-intensity, tactile stimulation (Waldenstrom et al. 2003) but the effect of segmental inhibition on the timing and outcome of the tuning period are not known. Descending inhibition plays a key role in the postnatal tuning of reflexes and behaviour: rats spinalised as neonates display inappropriate and disorganised nociceptive reflex receptive fields and behaviour that are reminiscent of those observed in the neonate, despite intact segmental mechanisms (Levinsson et al. 1999). It would therefore appear that segmental inhibition is not sufficient for determining the critical period for nociceptive processing in the dorsal horn, but it may still prove to be necessary.

A key novel finding in this thesis is that cutaneous inhibitory RFs of neonatal dorsal horn neurons show poor spatial tuning. It has been suggested above that this may act to counter some of the excitation produced by the large cutaneous excitatory RFs seen during the early postnatal period. This has been explicitly tested in neurons of the superior colliculus of the hamster which code for velocity tuning of visual stimuli. Chronic NMDAR blockade during development enlarges the excitatory RFs of these neurons; however, the strength and extent of inhibitory RFs was also increased to compensate and overall velocity tuning remained constant (Razak and Pallas 2005). Blockade of NMDARs in the dorsal horn from birth prevents the postnatal reduction in excitatory RF size (Beggs et al. 2002); it would be instructive to test whether this experimental manipulation similarly prevents the postnatal spatial tuning of cutaneous inhibitory RFs.

5.3.3 Potential mechanisms for activity-dependent homeostasis and plasticity

Advances in the understanding of activity-dependent homeostasis in the developing central nervous system have recently been driven by the model of synaptic scaling. This concept, now demonstrated empirically both *in vitro* and *in vivo* relies on the elegant idea that individual neurons should adjust over time to keep their overall firing rate constant, and thus avoid the instability that could accompany strengthening or weakening of synapses due to associative learning. If the firing rate for a cell becomes raised due to strengthening of a particular synapse [for example via long-term potentiation (LTP)], then synaptic scaling would dictate that all excitatory synapses onto the cell be reduced in proportion to their strength. This would return the firing rate to the baseline value, whilst preserving the information contained in the relative weights of the various synapses (Turrigiano et al. 1998; Desai et al. 2002; Turrigiano and Nelson 2004). The majority of research on synaptic scaling has concentrated on induction and expression at excitatory synapses, but the concept applies equally to inhibitory synapses, although the direction of change in synaptic strength would be reversed. Some studies have shown that synaptic scaling of inhibitory inputs does occur when

activity is blocked, but that decreases in synaptic strength are also accompanied by a reduction in the number of GABAergic synapses (Rutherford et al 1997; Kilman et al. 2002).

The Hebbian theory of associative learning states in its simplest form that "cells that fire together, wire together", whereby excitatory synapses between concurrently firing cells are strengthened via mechanisms such as LTP. This has recently been extended by the discovery of the importance of precise spike timing in determining the direction of change of synaptic weight: briefly, if firing in the presynaptic cell precedes firing in the postsynaptic cell by ~ 10 ms, then that synapse will be maximally strengthened. If firing in the postsynaptic cell precedes that in the presynaptic cell by a similar time, then the synapse will be considerably weakened. If the pre and postsynaptic spikes are separated by more than ~30 ms, then the synapse strength is unaltered (Zhang et al. 1998; Dan and Poo 2004). There is evidence that relative timing of action potentials is important for *in vivo* plasticity of, for example, receptive fields in sensory systems both during development and in the adult (Clark et al. 1988; Maffei and Galli-Resta 1990), and this spike timing dependent plasticity (STDP) could be the mechanism underlying this.

Activity-dependent modification of inhibitory synaptic strength should, however, be non-Hebbian as the desirable relationship between the pre and postsynaptic cells is specifically to not fire together (Pallas et al. 2006). Despite this, there have been reports of STDP of inhibitory synapses, in which GABAergic inhibition is weakened via a downregulation of KCC2 and subsequent positive shift in the chloride reversal potential when the pre and postsynaptic cells fire within 20 ms of each other (Woodin et al. 2003). Interestingly, and unlike STDP at excitatory synapses, the effect on GABAergic transmission is independent of the order in which the action potentials occur. It is therefore unclear how this activity-dependent change might relate to homeostatic regulation of firing. Furthermore, it has been reported that repeated postsynaptic firing can by itself downregulate KCC2 and cause depolarisation of the postsynaptic cell by GABA (Fiumelli et al. 2005). This would actually appear to produce destabilising positive feedback in the postsynaptic cell, although the cell-

autonomous synaptic scaling mentioned above may prevent this. However, in the bilateral hearing deprivation experiments mentioned in section 5.3.2, the compensatory decrease in inhibitory strength was mediated in part by a reduction in KCC2 and a positive shift in the chloride reversal potential (Vale et al 2003).

Changes in inhibition due to altered chloride homeostasis can therefore be induced in a number of ways, and may act both to balance excitation and inhibition and to perturb that balance, depending on the circumstances. In adult dorsal horn cells, GABA similarly becomes depolarising after peripheral nerve injury, and in this instance the change would appear to disrupt the balance of excitation and inhibition in the dorsal horn (Coull et al. 2003). It would be interesting to test whether deprivation of cutaneous input during the early postnatal period also produces a similar shift in the anion reversal potential in the adult, and whether such a shift would occlude that produced by later nerve damage.

5.4 Concluding remarks

The correct balance of inhibitory and excitatory connections is vital for the proper development and maintenance of circuitry in the central nervous system. In the neonatal spinal cord, I have demonstrated that GABAergic inhibitory transmission is functional but that the organisation of descending and segmental inhibitory circuits is nevertheless immature at this stage. The balance between inhibition and excitation appears to be maintained in the neonate at the level of the single cell, and the total inhibitory drive to a single cell does not change with age, as demonstrated by the similarity of the effect of gabazine at different ages. However, the spatial distribution of inhibition is greater in the young central nervous system, so the inhibition must in theory be more 'dilute' (see figure 5.1). Hence, a given peripheral or descending input to the dorsal horn may recruit less inhibition in the neonate than in the adult. In this scenario, the neonate is likely to show increased excitability of reflexes and behaviour in response to afferent and descending stimulation compared to the adult.

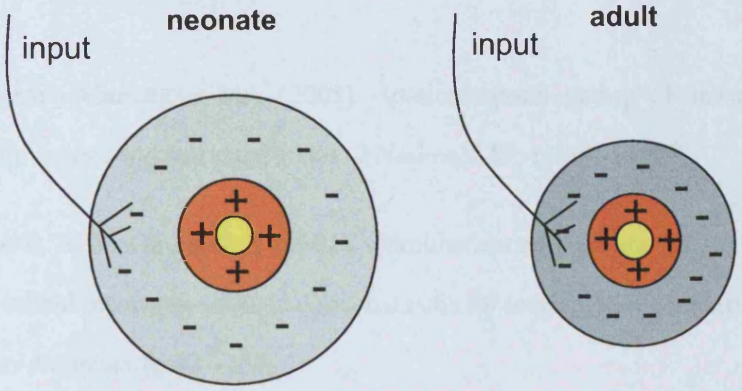


Figure 5.1 Representation of the hypothetical spinal regions providing inhibitory (blue) and excitatory (red) input to indiviual cells (yellow) in the neonate and the adult. A cell may have the same total inhibitory input in the neonate as the adult, but if the distribution of the inhibition is more widespread then a given input will recruit a smaller proportion of the inhibition.

Further experiments are required to investigate the effects of increased or decreased sensory input during the postnatal period on the balance of excitation and inhibition in the dorsal horn. If the critical period for development of reflexes reflects a critical period in the wiring of the dorsal horn, this could have important clinical implications. Premature infants often undergo repeated painful procedures which presumably cause abnormal sensory load. This may lead to activity-dependent plasticity and long-lasting changes in dorsal horn circuits. Furthermore, the possibility that the timing of circuit development may depend on GABAergic activity and may be perturbed by benzodiazepines, as is the case in parts of the visual system, could have consequences for the administration of this sedative in very young infants. A full understanding of inhibitory mechanisms in the developing spinal cord is important for the appropriate care of young infants during painful procedures, and may provide clues for the management of chronic pain in adults.

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